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ORIGINAL PAPER

Serum protein profiling and proteomics in autistic spectrum disorder using magnetic bead-assisted mass spectrometry

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Abstract The pathophysiology of autistic spectrum disorder (ASD) is not fully understood and there are no diagnostic or predictive biomarkers. Proteomic profiling has been used in the past for biomarker research in several non-psychiatric and psychiatric disorders and could provide new insights, potentially presenting a useful tool for generating such biomarkers in autism. Serum protein pre-fractionation with C8-magnetic beads and protein profiling by matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-ToF-MS) were used to identify possible differences in protein profiles in patients and controls. Serum was obtained from 16 patients (aged 8–18) and age-matched controls. Three peaks in the MALDI-ToF-MS significantly differentiated the ASD sample from the control group. Sub-grouping the ASD patients into children with and without comorbid Attention Deficit and Hyperactivity Disorder, ADHD (ASD/ADHD+ patients, $n = 9$; ASD/ADHD− patients, $n = 7$), one peak distinguished the ASD/ADHD+ patients from controls and ASD/ADHD− patients. Our results suggest that altered protein levels in peripheral blood of patients with ASD might represent useful biomarkers for this devastating psychiatric disorder.

Keywords Autism · Biomarker discovery · Serum profiling · Proteomics

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Introduction

Autistic spectrum disorder (ASD) is a complex brain disorder characterised by specific impairments, defined by classification systems such as DSM-IV and ICD10 [2, 38]. The main impairments lie in the domains of social interaction, language, communication and imaginative play. To date, the etiopathogenesis of ASD is poorly understood, but scientific evidence supports the notion of a neurodevelopmental disorder, since autism is often associated with diseases with neural maldevelopment [2, 30, 35]. Furthermore, post-mortem brain and structural and functional MRI studies indicate cytoarchitectural and morphological abnormalities in the brainstem and cerebellum, in the limbic system and the cortex of patients [20, 35]. While genetic approaches have largely dominated neuropsychiatric research in recent years, proteomic techniques are increasingly evolving as additional tools for elucidating the pathophysiology of mental disorders. Proteomics facilitates the study of many pathways and complex interaction networks at the protein level, thereby potentially being "closer" to the underlying pathophysiological processes. By using proteomic tools, it is possible to identify quantitative and qualitative protein patterns in a wide variety of tissues, in order to establish specific diagnostic and prognostic biomarkers.

To date, the diagnosis of autism is solely based on the patient's history and the observation of behavioural abnormalities; valid disease markers are not available. A reliable protein biomarker, however, could significantly contribute to an early and more exact ASD diagnosis, a crucial prerequisite for an early behaviour-modifying therapeutic intervention. Furthermore, a diagnosis at an early stage could contribute to developing better coping strategies within families confronted with classical ASD features of a child's behaviour. It has been suggested that molecules involved in serotonin metabolism [10, 11, 31] and several cytokines and chemokines [17, 29, 34, 39, 45] are associated with ASD. Altered blood levels of neurotrophic factors have also been reported [28, 32, 33]. Recently, Corbett and co-workers [8] conducted a proteomic study in autism, screening a group of autistic children for differentially expressed serum proteins. Here we present an alternative proteomic approach, using different methods in order to primarily determine a protein pattern that can be used as biomarker, rather than to identify specific differentially expressed proteins. In contrast to Corbett et al. [8] we have analysed whole proteins—not peptides after tryptic digest—using MALDI-ToF-MS.

Patients and methods

Participants

Sixteen male patients with ASD (age range from 8 to 18 years) and 16 male healthy control subjects participated in this study. There was no significant age difference between patient and control group ($P = 0.98$). The demographic statistics and ASD ICD-10 diagnoses of the patients group are presented in Table 1. A priori, all subjects and parents had given written informed consent. The study was approved by the ethics committee of the University of Würzburg.

Diagnoses and psychometry

For the index group, the inclusion criteria consisted of a diagnosis of ASD based on the ICD-10/DSM-IV criteria determined by a child psychiatrist. The ASD diagnosis was further confirmed by the following questionnaires: FSK (German version of the Social Communication Questionnaire, SCQ [3, 37]), Autism Diagnostic Interview-Research, ADI-R (German version: ADI-R—Diagnostisches Interview für Autismus—Revidiert [4, 25]) and Autism Diagnostic Observation Schedule, ADOS [24] (German version: ADOS—Diagnostische Beobachtungsskala für Autistische Störungen [36]). Intellectual ability was measured as previously described [40, 41]. For the ASD questionnaire cut-offs, the mean scores and intellectual ability in our ASD group see Table 1. The control group all attended primary, secondary or grammar school, suggesting average IQ ranges. To assess the social competence and behavioural problems in the control group, the Achenbach Child Behaviour Check List (CBCL) was used [1]. All participants were screened via parental interview for current and chronic physical illness. In the autism group, children and adolescents with a known severe somatic or neurological disorder as well as schizophrenic psychoses were excluded from the study. Individuals were excluded from the control group if they suffered from a somatic and neurological disease, were taking medication or exhibited abnormal CBCL scores. For the comorbid diagnoses in the ASD group see Table 1. The following medication was used in ASD patients: nine children took an ADHD medication such as amphetamine, methylphenidate, slow-release forms of methylphenidate, or atomoxetine. One child received fluoxetine, another tiapridex in addition to the stimulant medication. An adolescent had a fluoxetine monotherapy, another was on risperidone. One child was treated with a sertraline/lithium co-medication.

Serum sample collection and protein purification

Blood samples were collected, allowed to clot for 30 min at room temperature and then centrifuged at 3,000 rpm for 5 min, aliquoted (500 µl) and stored at −80°C until analysis. Each patient sample was matched with a control sample and each pair was processed simultaneously. Magnetic beads with C8-functionality (C8-purification kit MB-HIC, Bruker Daltons, Bremen, Germany) were used for protein isolation according to the manufacturer's instructions except a doubling of the elution volume recommended. 8 µl of the eluate was mixed with 8 µl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile/0.1% TFA). 4 µl of this mixture was spotted in quadruplicate on the MALDI plate.

Protein profiling using matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-ToF-MS)

MALDI-ToF-MS measurements were performed using a Voyager DE-STR mass spectrometer (Applied Biosystems, Warrington, UK), operating in positive, linear mode. Spectra were collected after external calibration and a truncated mass range from 1 to 12 kDa as well as 12–70 kDa was considered for peak selection, with a laser intensity of around 2,100–2,300, a delay time of 400 ns and an acceleration voltage of 25 kV. Spectra were collected automatically with accumulation of 600 laser shots per spectrum using the following acceptance criteria: minimal

Table 1 Demographic statistics of ASD patients

Male patients (*n*)	16
Age (years), mean ± SD	13.6 ± 3.5
ASD ICD 10-diagnoses (*n*)	
F84.0 Childhood autism	2
F84.1 Atypical autism	5
F84.5 Asperger's syndrome	9
Comorbid ICD 10-diagnoses (*n*)	
F31.3 Bipolar affective disorder, current episode mild or moderate depression	1
F42.2 Mixed obsessional thoughts and acts	1
F80.0/F81.1 Specific speech articulation disorder/expressive language disorder	1/1
F83 Mixed specific developmental disorder	1
F90.0/90.1 Disturbance of activity and attention/hyperkinetic conduct disorder	8/1
F93.3 Sibling rivalry disorder	1
F94.0 Elective mutism	1
F95.1/F95.2 Chronic motor or vocal tic disorder/combined vocal and multiple motor tic disorder	1/1
F98.0/F98.1 Non-organic enuresis/encopresis	1/1
Psychiatric co-medication (patients, *n*)	
Methylphenidate (including Medikinet ret.®, Concerta®)	6
Amphetamine	2
Atomoxetine	1
Fluoxetine	2
Sertraline	1
Lithium acetate	1
Risperidone	1
Tiapridex	1
Autistic features (score ± SD)	
FSK	19.9 ± 6.1
ADOS sum of communication and social interaction (autism cut-off = 10, autistic spectrum disorder = 7)	11.5 ± 4.4
ADI-R social interaction (cut-off 10)	19.9 ± 8.4
ADI-R communication and language (cut-off for speaking children = 8)	15.0 ± 4.9
ADI-R repetitive behaviours and stereotyped pattern (cut-off = 3)	5.8 ± 3.1
ADI-R abnormal development before the age of 3 years (cut-off = 1)	2.5 ± 1.7
Intellectual ability (patients, *n*)	
IQ 70-84	7
IQ 85-115	6
IQ 116-129	3

ADI-R Autism diagnostic interview-research, *ADOS* autism diagnostic observation schedule, *ASD* autistic spectrum disorder, *FSK* Fragebogen zur Sozialen Kommunikation, original version: social communication questionnaire (SCQ)

signal intensity 10,000, maximal signal intensity 55,000 (local base peak). The average of the quadruplicate spots was combined to represent each sample and individual samples of poor spectral quality were excluded from calculating the average spectrum.

Data processing using Data Explorer 4.0 and MarkerView™ software

The unprocessed spectra were analysed using Data Explorer 4.0 (Applied Biosystems, Warrington, UK). For the truncated spectra (1–12 and 12–70 kDa), five standard deviations of noise removal, a noise filter with a correlation factor of 0.7 and a Gaussian smooth with a filter width of 5 points were applied. The 1–12 kDa spectra were further truncated (2.5–6.7, 6.3–9.5, 9–11.5 kDa) and each truncated spectrum was calibrated for a second time by two peaks that were clearly present in all patient and control spectra to increase *m/z* accuracy. The 12–70 kDa spectra were calibrated with three omnipresent peaks. All peak lists were then imported into the MarkerView™ software (Sciex Corp, Toronto, Canada) which performed peak

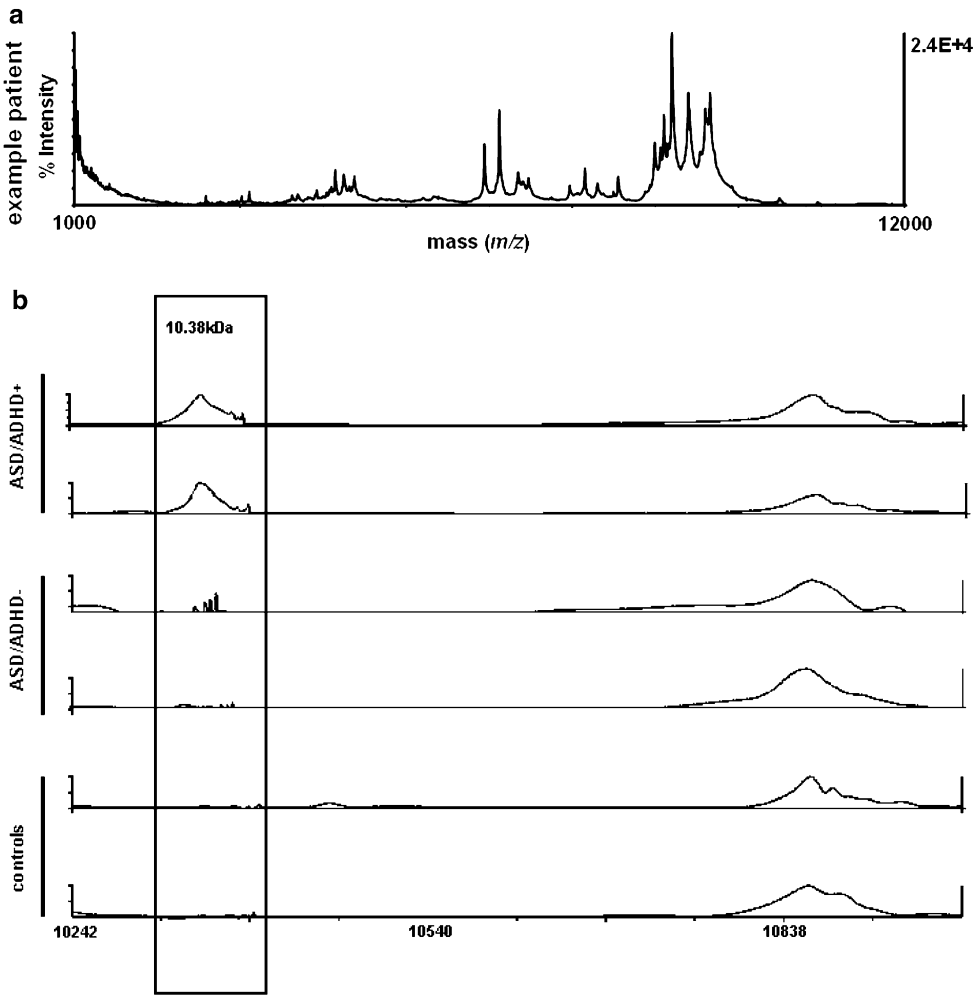

Fig. 1 **a**. Representative example of a 1,000–12,000 Da patient MALDI-MS spectrum. **b** MALDI-MS spectra of two ASD/ADHD+, two ASD/ADHD− patients and two controls. The ASD/ADHD+ patients show a clear peak with a *m/z* ratio of 10.38 kDa

alignment, using a mass alignment tolerance of 4,000 ppm, a minimum required response of 10 and a maximum number of peaks of 5,000. Samples were normalised by adjusting the intensity values by a run-specific constant.

Statistical analyses

Within the MarkerView™ software, the sixteen patient samples were defined as one group, while the sixteen control samples represented the second group. In a second calculation, the patient group was divided into: (1) ASD patients with comorbid ADHD (ASD/ADHD+ patients, $n = 9$), (2) ASD patients without comorbid ADHD (ASD/ADHD− patients, $n = 7$). We performed the entire protein profiling, data processing and statistical analysis as a duplicate experiment. Since the peak intensities were not normally distributed, we analysed the ranked data using the Mann–Whitney *U* test. This was the conservative test, and since test across peaks were clearly correlated, additional corrections for multiple comparisons were not performed.

Results

Data processing with MarkerView™ Software revealed a peak with the *m/z* ratio of approximately 10.38 kDa which differentiated significantly between the whole ASD patient and control group ($P = 0.008$) with a higher peak area in the patients group (fold change = 3.55). We performed a second calculation to compare ASD/ADHD+ and ASD/ADHD− patients and found the 10.38 kDa peak significantly different in the ASD/ADHD+ and ASD/ADHD− groups ($P = 0.023$) with a higher peak area in ASD/ADHD+ patients (fold change = 4.15). This 10.38 kDa peak did not distinguish between ASD/ADHD− patients and the matched controls ($P = 0.48$). However, the 10.38 kDa peak distinguished significantly between ASD/ADHD+ patients and the corresponding controls ($P < 0.001$, fold change = 5.48). Figure 1a shows a representative example of a 1,000–12,000 Da patient MALDI-ToF-MS spectrum after base line correction, Gaussian smooth and noise filtration/removal. In Fig. 1b, spectra regions around the 10.38 kDa peak of two ASD/ADHD+ patients, two ASD/ADHD− patients and two

Table 2 Peaks (*m/z* ratios) with different peak areas in ASD and control group

	Significance	Fold change
Peak 10.38		
Whole ASD versus whole control group	**0.008**	3.55
ASD/ADHD+ versus ASD/ADHD−	**0.023**	4.15
ASD/ADHD− versus matched controls	0.480	
ASD/ADHD+ versus matched controls	**<0.001**	5.48
Peak 5.15		
Whole ASD versus whole control group	**0.010**	−9.53
ASD/ADHD+ versus ASD/ADHD−	0.380	
ASD/ADHD− versus matched controls	0.140	
ASD/ADHD+ versus matched controls	0.200	
Peak 4.40		
Whole ASD versus whole control group	**0.056**	−2.49
ASD/ADHD+ versus ASD/ADHD−	0.370	
ASD/ADHD− versus matched controls	0.034	−3.75
ASD/ADHD+ versus matched controls	0.100	−2.01

Fold change: down (−) indicates a smaller peak area in the ASD group compared to the control group

healthy controls are shown. The 10.38 kDa peak is clearly visible in the ASD/ADHD+ group compared to the control and ASD/ADHD− group. In contrast to the varying 10.38 kDa peak, the adjacent 10.85 kDa peak was consistent.

Another peak, *m/z* 5.15 kDa, differentiated significantly between the complete ASD patients and the control group ($P = 0.010$) with a higher peak area in the control group (fold change = −9.53); Table 2 provides detailed data for all peaks which differ in their area between the ASD and the control group. In a second calculation run, however, the 5.15 kDa peak area did not differ between ASD/ADH and ASD/ADHD− patients ($P = 0.380$). MarkerView™ Software revealed another 4.40 kDa peak that differentiated between the whole ASD patient group and the controls ($P = 0.056$). This peak showed a significantly higher peak area in the control group (fold change −2.49) and did not differ between the ASD/ADHD+ and ASD/ADHD− groups, but separated significantly the ASD/ADHD− from the matched controls ($P = 0.034$, fold change −3.75; see Table 2). Spectrum regions around the 5.15 kDa peak (Fig. 2a) and around the 4.40 kDa peak (Fig. 2b) are presented for two patients and two controls. Although the sample sizes were rather small in this study, the design was clearly of sufficient power to detect fold change in the region of at least 2.5.

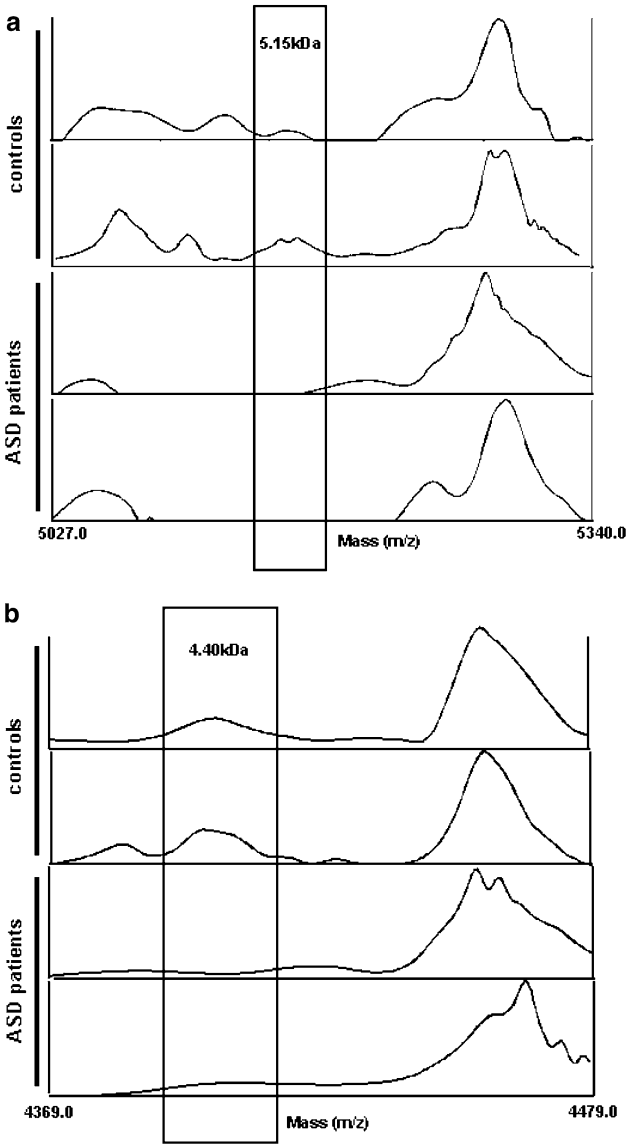

Fig. 2 **a** MALDI-MS spectra of two ASD patients and two controls. The control persons show a peak with a *m/z* ratio of about 5.15 kDa compared to the ASD patients. **b** MALDI-MS spectra of two ASD patients and two controls. The control persons show a peak with a *m/z* ratio of about 4.40 kDa compared to the ASD patients

Discussion

The analysis of specific biological entities for biomarker evaluation in autism shows promise [10, 11, 17, 28, 29, 31, 33, 34, 39, 45] yet only one previous study, has studied the proteome for such biomarkers [8]. Our study undertook intact-protein proteomic assessment of serum protein patterns to reveal such biomarkers. After pre-fractionation, mass spectra were gained with MALDI-ToF-MS. Data processing and subsequent data analysis revealed three peaks with *m/z* ratios of approximately 4.40, 5.15 and 10.38 kDa that were significantly different between the ASD and control sample.

The proteome of the blood contains highly abundant proteins and also a low molecular weight (LMW) fraction that includes several classes of proteins [5, 42]. In our study the three potential biomarker peaks all showed *m/z*

ratios <11 kDa, agreeing with previous reports of potential biomarkers in the LMW section of the blood proteome for diverse diseases [5, 12, 13, 15, 26, 42, 44]. The results of our study support previous findings of a differentiated regulation of different pathways in the blood of ASD patients including serotonin [6, 7, 9, 14, 26, 27, 42, 43] norepinephrine [21–23] and neurotrophic factors [16, 17, 28, 32, 33]. In one of the first proteomic studies conducted in autism, only subtle changes in the concentration of apolipoproteins in the blood were reported [8]. Our three differential peaks, in contrast, show higher fold changes (3.55, −2.49, and −9.53) between the ASD and control group. The aim of this study was to establish an easily manageable, clinically applicable method to determine protein patterns representing potential biomarkers. We did not primarily aim at specifically identifying the corresponding molecules. Desiring a fractionation method that requires only small sample quantities and can be automatised, we opted for serum proteome pre-fractionation with C8-magnetic beads. To our knowledge, this was the first use of this novel and facile technology in psychiatric research, in contrast to other labour-intensive fractionation methods this approach allows for the automated analysis of many clinical samples within a short period of time. Previously, this method had exclusively and successfully been used in research projects focusing on somatic disorders Furthermore, intact proteins are studied rather than tryptic digests; thus, no variation are introduced by the inclusion of the enzyme-mediated digestion step.

We used for the first time in psychiatric biomarker research a serum profiling method based on the combination of C8-magnetic beads and MALDI-ToF-MS. We were able to identify three peaks with significantly different expression patterns between patients and controls. Our findings contribute to the use of proteomics in biomarker research and suggest a protein profile potentially representing disease markers for ASD in peripheral blood.

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