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Urinary opioid peptides in children with autism spectrum disorders. A Pilot Study

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Abstract.
BACKGROUND: Circulating β-casomorphins (BCM) and gluten exorphins (GE), which are exogenous opioid peptides originating from bovine milk protein casein and cereal protein gluten, respectively, have been proposed as potential trigger factor to symptoms observed in children with autism.
OBJECTIVE: Given the debate surrounding the detectability of these opioid peptides in body fluids, particularly using highly sensitive and specific mass spectrometry (HPLC-MS) techniques, we aimed to investigate their presence in urine samples of autistic subjects.
METHODS: We employed an HPLC-MS method for peptide detection.
RESULTS: The presence of several BCMs and GEs in the urine of both autistic children (ASD) and healthy controls (HC) was documented. The detection of dietary opioid peptides even at very low concentrations underscores the sensitivity of this novel HPLC-MS method. BCM-8 was more often detected in the ASD group compared to the HC group. A higher prevalence of gastrointestinal (GI) symptoms were also observed in the ASD group.
CONCLUSIONS: This pilot study supports the presence of BCMs and GEs in urine samples in subjects with autism as well as healthy controls which was the main goal of this pilot study. Prolonged exposure to bovine BCMs and GEs may play a role in the manifestation of core and GI symptoms in subgroups of autism. Further research is warranted to investigate this phenomenon thoroughly.

Keywords: Autism, opioid peptides, gluten and milk, Mass spectrometry

1. Introduction

Autism spectrum disorders (ASD) is a group of neurodevelopmental disorders that involve definite impairments in social interactions, disturbance in language, and a stereotyped pattern of behavior. According to the literature [1], the prevalence of ASD is estimated to be between 1–2%, making it a significant public health concern [1]. Importantly, these core symptoms often co-occur with a range of neurological and psychiatric comorbidities, including epilepsy, intellectual disabilities,

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ADHD, sleep disturbances, eating issues, self-injurious behavior, and notably, gastrointestinal problems [2–6]. This diversity of symptoms underscores the heterogeneity of children with autism, revealing ASD as a spectrum of neurobiological disorders with overlapping phenotypes and various etiological underpinnings.

Twin studies have provided substantial evidence, indicating a high concordance rate (60–90%) among monozygotic twins compared to a significantly lower rate among dizygotic twins. This high concordance rate strongly suggests a substantial genetic influence on ASD [7]. Nevertheless, the exact etiology and pathogenesis of ASD remain largely elusive. For most children with ASD, no single underlying cause can be identified, and there is a dearth of evidence-based biomedical investigations. The genetic mechanisms responsible for ASD are not fully understood, and we are confronted with a wide array of biochemical abnormalities and potential toxicological factors that may contribute, either individually or in combination with genetic vulnerabilities, to the manifestation of the ASD phenotype.

One long-standing hypothesis in ASD research is the opioid peptide excess [8–11]. The opioid peptide excess theory postulates that exogenous opioid peptides, which exhibit morphine-like activity, are derived from various dietary sources such as dairy and gluten-containing cereals. Dietary peptides are recognized as essential sources of exogenous opioids due to their structural similarities to endogenous opioids. The opioid excess hypothesis suggests that a combination of reduced concentrations and activity of digestive enzymes, including proteases and peptidases like the DPPIV enzyme [12, 13], along with increased intestinal permeability [14–16], may result in an elevated presence of biologically active opioid peptides from food sources. These dietary opioid peptides will not be produced endogenously; hence the level of detected peptides will also depend on the amount of milk protein and gluten consumed in the diet.

The main goal of the present pilot study was to determine whether opioid peptides from food could be detected in the urine applying a novel HPLC-MS analysis in a group of clinically well-characterized children suffering from ASD and compare with healthy children (HC).

2. Ethics

Written informed consent was obtained from the parents of all study participants. The pilot study was approved by the Regional Committees for Medical and Health Research Ethics (REK). The study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964).

3. Materials and methods

Study design: This study is a pilot study conducted as a case-control study.

3.1. Patients included and clinical tools used

Children with ASD were identified by investigating patients who were received at child habilitation services, Innlandet Hospital Trust, Lillehammer Norway. Eighteen patients with an ASD diagnosis agreed to participate in the study. They had been diagnosed using the gold standard in ASD diagnostic procedures with ADOS (Autism Diagnostic Observation Schedule) and ADI-R (Autism Diagnostic Interview) and underwent a clinical medical examination in accordance with guidelines prepared by Southern and Eastern Norway Regional Health Authority. The medical investigation included the medical and developmental history and a physical examination. The medical and developmental histories focused on the family history, birth characteristics, medical co-morbid symptoms, early

Table 1
Children with autism and gastrointestinal symptoms (GIT). F84.0 Childhood autism, F84.9 ASD disorder not otherwise specified, BCMs- β-casomorphins

ID number	Age	Sex	Diagnoses	GIT symptoms	Diet	BCMs
1	5 y	M	F84.0	Constipation	No	No
2	7 y	F	F84.0	Constipation	No	Yes
4	5 y	M	F84.0	Diarrhea, constipation, vomiting	Yes	Yes
5	4 y	F	F84.0	Constipation diarrhea	Yes	Yes
6	4 y	F	F84.0	Constipation, abdominal pain	No	Yes
9	5 y	M	F84.0	Constipation, diarrheaAbdominal pain	No	Yes
11	4 y	F	F84.0	Constipation, abdominal pain	No	Yes
14	4 y	M	F84.0	Abdominal pain	No	Yes
33	5 y	F	F84.9	Constipation, abdominal pain	No	Yes

Children with autism and gastrointestinal symptoms (GIT). F84.0 Childhood autism, F84.9 ASD disorder not otherwise specified, BCMs-β-casomorphins.

development, age and nature of symptom onset, sleep history, eating pattern, GI symptoms, and current psychiatric and other disorders.

The physical examination included assessment of the somatic status and identifying any problems by recording the general status of the subjects (height, weight, head circumference, and vision and organ statuses), as well as performing a neurological examination (dysmorphic signs, gross and fine motor skills, coordination, reflexes, hearing function, and skin characteristics). Additional investigations included blood tests with a chromosome investigation (using microarray-based comparative genomic hybridization) and metabolic screening of the urine, EEG and cerebral MRI were applied when specific indications were present.

The urine samples were collected from these 18 children (14 boys and 4 girls) with age between 3 and 10 years (Mean 7.6 years). The patients underwent clinical evaluations and medical examinations samples were obtained from April to June 2022 at the pediatric outpatient clinic at Lillehammer Hospital. 17 patients were classified as having childhood autism (F84.0); the remaining patient was diagnosed as ASD disorder not otherwise specified (F84.9). Among the 18 investigated ASD patients, 9 showed clinical signs of intellectual disability (ID). Comorbid conditions such as ADHD were found in one patient. Epilepsy was diagnosed in one patient and pathological EEG were found in two patients.

GI symptoms in the patient group were reported by parents and children. Abdominal pain, constipation, chronic diarrhea, or vomiting problems were reported by 9 (47%) of the 18 ASD patients (Table 1). Two of the 18 children were on the diet without milk and gluten.

4. Control subjects (Healthy controls – HC)

Eighteen non-autistic children were included as controls, comprising 10 boys and 8 girls with age between 3 and 17 years (Mean 10.6). The children visited the Department of Pediatrics, Innlandet Hospital Trust for different kinds of medical conditions. They were all admitted to the same outpatient clinic by their general practitioner or pediatrician. They were not interviewed in depth and were not neurologically examined. The exclusion criteria for the control group included neurological and neurodevelopmental diseases or suffering from immune insufficiency and gastrointestinal (GI) symptoms. Children in the control group did not have ADHD or mild intellectual disability (ID) diagnoses and were from the same area as the patients with similar characteristics. They were selected to the study in a similar period of year, their urine tests were mostly taken in the same part of the day, as the subject group.

The mean age in the ASD group was 7.6 years (SD 2.7) and 10,6 years (SD 3.9) in the HC group ($p = 0.003$). The observed significant age difference between the two groups is important to consider when interpreting the study results and will be discussed in the Discussion part of this paper.

4.1. Urine collection and sampling – Preanalytical aspects

First morning urine was collected for all patients and healthy controls and 10 mL urine was transferred within 5 minutes to a Monovette tube (Sarstedt AG & Co, Nümbrecht, Germany). The tubes contain a peptidase inhibitor to inhibit fast degradation of the peptides. This is a very crucial step in the preanalytical procedure since the level of opioid peptides are low and will be degraded very fast in biological samples like urine and blood. The urine was mixed for 10 s with the inhibitor, and thereafter the samples were stored in freezer (–20°C) until analysis. All samples were coded allowing analysis to be carried out blind to diagnostic status. The codes were opened after the results from the HPLC-MS investigation were reported. Urine was thawed prior to the solid phase extraction (SPE). A standard volume of urine was added to a standard volume of Internal standards (IS) for all 13 peptides according to the method. The urine samples were applied to the SPE and after conditioning of the SPE, washing and elution, the samples were ready for the HPLC-MS analysis.

4.2. High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis

For all urine samples, the peptides were separated by HPLC chromatography (Agilent Technologies, Santa Clara, CA, United States) using a reversed phase peptide column (Agilent Technologies) with a pre-column guard (Agilent Technologies). The analysis time was 25 minutes, and a gradient elution was developed. After chromatography the peptides were further analyzed by electro spray ionization (ESI) mass spectrometry (Triple Quadrupole MS – Agilent Ultivo) to determine the peptides. MRM (Multiple Reaction Monitoring) transitions were measured for all ions, based on the mass of the molecular ion as precursor ion and two fragment ions as respectively quantifier and qualifier ions. The MRMs for the 13 selected peptides and corresponding internal standards were selected based on analysis on Agilent MassHunter Workstation Optimizer for Ultivo LC/TQ, Version 1.2. For Qualitative Analysis Agilent MassHunter Qualitative Analysis 10.0, for Data Acquisition Agilent MassHunter Acquisition Console and for Quantitative Analysis Agilent MassHunter Quantitative Analysis 10.2. were used. This method is a modification from the previous method described elsewhere [17].

4.3. Chemicals, peptides, and Internal Standards (IS)

Peptide standards, bovine BCM-3, BCM-4, BCM-4 amide, BCM-5, BCM-5 amide, BCM-7, and BCM-8 and GE-A4, GE-A5, GE-B4, GE-B5, GE-C, gliadorphin-7, and IS for each peptide (isotope labelled) were purchased from Pepmic, Suzhou, China with > 99% purity. Chemicals (Acetonitrile grade MSMS, FA grade MSMS) was ordered from VWR/Avantor, Oslo, Norway. The SPE was a Polymeric Reversed Phase 96-well plate (Phenomenex, Torrance, USA).

4.4. Statistics

The statistical analysis was performed by IBM SPSS Statistics v. 28.0.1.1 (IBM Corp.) using the Fisher's exact test.

5. Results

Urine from 18 cases with ASD and 18 healthy controls (HC) were sampled and analyzed. Taken as a whole, the ASD group shows no significant differences in the concentration of the investigated peptides compared to the HC group (Table 2). When studying the occurrence/frequency of detected peptides in the two groups, we found that, when considering all the peptides together, only a significant increased BCM-8 in the autism group ($p < 0.05$, Fisher's exact test) (Table 3) was found. The reason why we did not find other BMC and GE of significance in the urine besides BCM-8 is uncertain. Low concentration of the other peptides, coupled with the method's detection limit (LOD) not being sufficiently low to detect them, could be one contributing factor. Other reasons could be separation issues, method optimization and sample handling. This must be addressed in future studies.

6. Discussion

This study included a small number of patients simply because it was meant like a pilot study. We were able to detect peptides in urine at very low concentrations (pg/mL) using a highly sensitive method, which was the main goal of our pilot study. The urinary opioid peptide concentration did not reach statistically significant results when comparing the autism group to the control group. However, autistic children more frequently exhibited specific BCM-8 detection compared to the healthy controls ($p < 0.05$). According to our knowledge, there are no studies that can determine whether elevated levels of opioid peptides lead to more severe autism and gastrointestinal symptoms. The increase in opioid peptides is likely secondary or one of many contributing factors. Autism is a condition with a complex etiology. The questions are interesting, but to ascertain this, a much larger patient cohort is necessary. The same applies if we are to elucidate which symptoms correlate with elevated opioid peptides. It is beyond our capability to provide an answer based on this small pilot study. The primary goal of this pilot study was to determine whether there is indeed an increase in opioid peptides in a patient population comprising children with autism. It is worth mentioning that a single result with $p < 0.05$ could easily arise by chance at the 5% level when multiple tests were performed. BCM-8, as an opioid agonist with the potential to bind to μ-opioid receptors in the gastrointestinal tract and the brain, is of particular interest. BCMs, in general, are known to delay gastric emptying and inhibit intestinal transit by reducing the frequency and amplitude of intestinal contractions [18, 19]. Notably, in our study, 47% of the ASD children reported gastrointestinal (GI) symptoms, while no children in the HC group exhibited these symptoms. Specifically, eight out of nine ASD children experiencing constipation were found to have detected BCMs, a combination that was absent in the healthy group, in line with the exclusion criteria for the HC group. This indicates a significant difference ($p < 0.05$) in the occurrence of gastrointestinal symptoms and constipation between the autism patient group and the healthy control group and the detection of BCMs. The result indicates a significant association between the occurrence of gastrointestinal symptoms and the detection of beta-casomorphin peptides in urine among autism patients.

There was a statistically significant difference in age between the two groups ($p = 0.003$, Independent *t*-test). Whether this difference may impact the result is uncertain, as there is no data to indicate any age-related changes in opioid peptides. Age can be a confounding factor and may influence the outcomes being studied. The age gap between the ASD and HC groups may have clinical implications. It could reflect differences in developmental stages or the progression of symptoms between individuals with ASD and typically developing individuals. Clinicians and researchers should be aware of these age-related variations when designing interventions or conducting assessments. While the age difference is statistically significant, further investigation is warranted to explore its clinical relevance. Future

Table 2
A) Quantitative results for all investigated peptides in the 18 patients in the ASD group, and B) same for the control group

A)																		
Peptides	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14	Patient 15	Patient 16	Patient 17	Patient 18
GE-A5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-A4	73,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-B4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-3	0,0	0,0	0,0	0,0	70,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	110,0
BCM-5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1161,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-5 amide	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-4 amide	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Gliadorphin-7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-C	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-B5	150,0	36,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	363,0	0,0	0,0	0,0	0,0	0,0
BCM-7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-8	0,0	229,6	0,0	30,9	102,9	20,1	129,5	248,2	128,0	116,6	143,1	110,0	149,1	100,0	120,6	0,0	95,1	70,3
B)																		
Peptides	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Control 7	Control 8	Control 9	Control 10	Control 11	Control 12	Control 13	Control 14	Control 15	Control 16	Control 17	Control 18
GE-A5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-A4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-B4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-3	0,0	83,5	0,0	25,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	191,5	58,5	81,0	168,0
BCM-5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	185,5	0,0	0,0	0,0	0,0	0,0	314,5	0,0	0,0	0,0
BCM-5 amide	0,0	0,0	0,0	0,0	28,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-4 amide	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Gliadorphin-7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-C	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
GE-B5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	715,5	0,0	421,5	0,0	0,0	0,0	0,0	0,0	0,0
BCM-7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
BCM-8	202,3	54,9	0,0	124,8	104,7	0,0	0,0	189,1	168,1	0,0	0,0	0,0	0,0	214,0	46,7	53,5	0,0	0,0

Table 3
Numbers of patients (ASD) and controls (HC) with detected BCM-8 versus without detected BCM-8 (No BCM-8 Detected), $p < 0.05$, Fisher's exact test

	BCM-8 Detected	No BCM-8 Detected	Total
Autism Group (ASD)	15	3	18
Healthy Control (HC)	9	9	18
Total	24	12	36

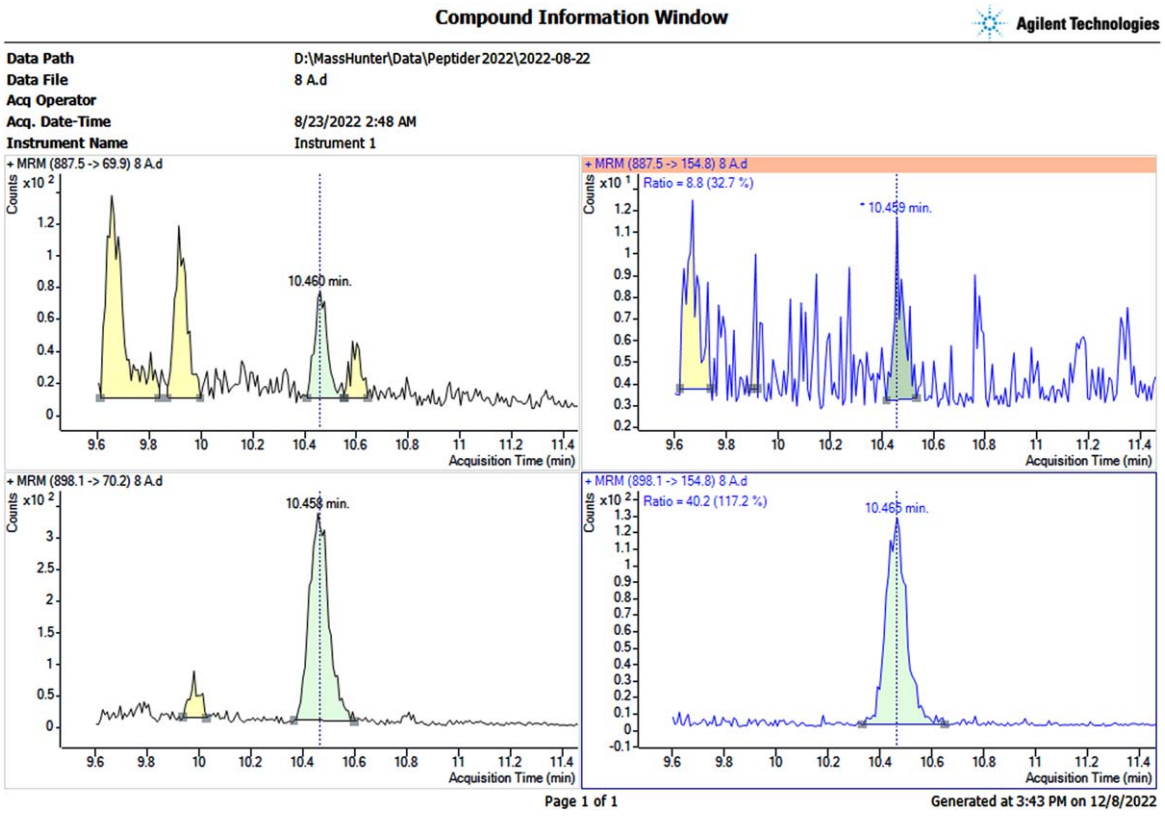

Fig. 1. Detected BCM-8 in one ASD patient with Multiple Reaction Monitoring (MRM) (upper 2 illustration) and Internal Standard (lower 2 illustration) (extracted from Agilent MassHunter Quantitative Analysis 10.2).

studies could delve deeper into how age influences the manifestation of symptoms and the presence of peptides in urine among individuals with ASD.

7. Conclusions

Our main goal was to investigate if dietary opioid peptides are detectable in urine based on a novel LC-MS method. In our pilot study with few participants, we did not observe a significant increase in peptide concentration in the autism group compared to the control group. However, we did identify a heightened occurrence of BCM-8 in children with ASD when compared to their healthy counterparts. To advance our understanding of the potential link between opioid peptides and autism and gastrointestinal symptoms, larger, meticulously designed, and high-quality clinical trials are imperative. It is our opinion

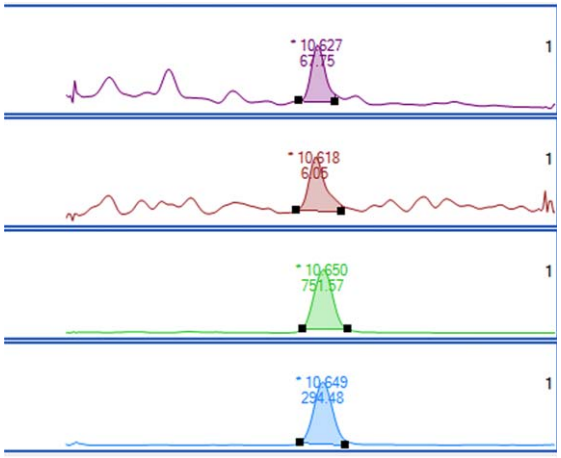

Fig. 2. Detected BCM-8 in another ASD patient with two MRMs (2 upper row) and two Internal Standards (IS) MRMs (2 lower row) (extracted from Agilent MassHunter Qualitative Analysis 10.0).

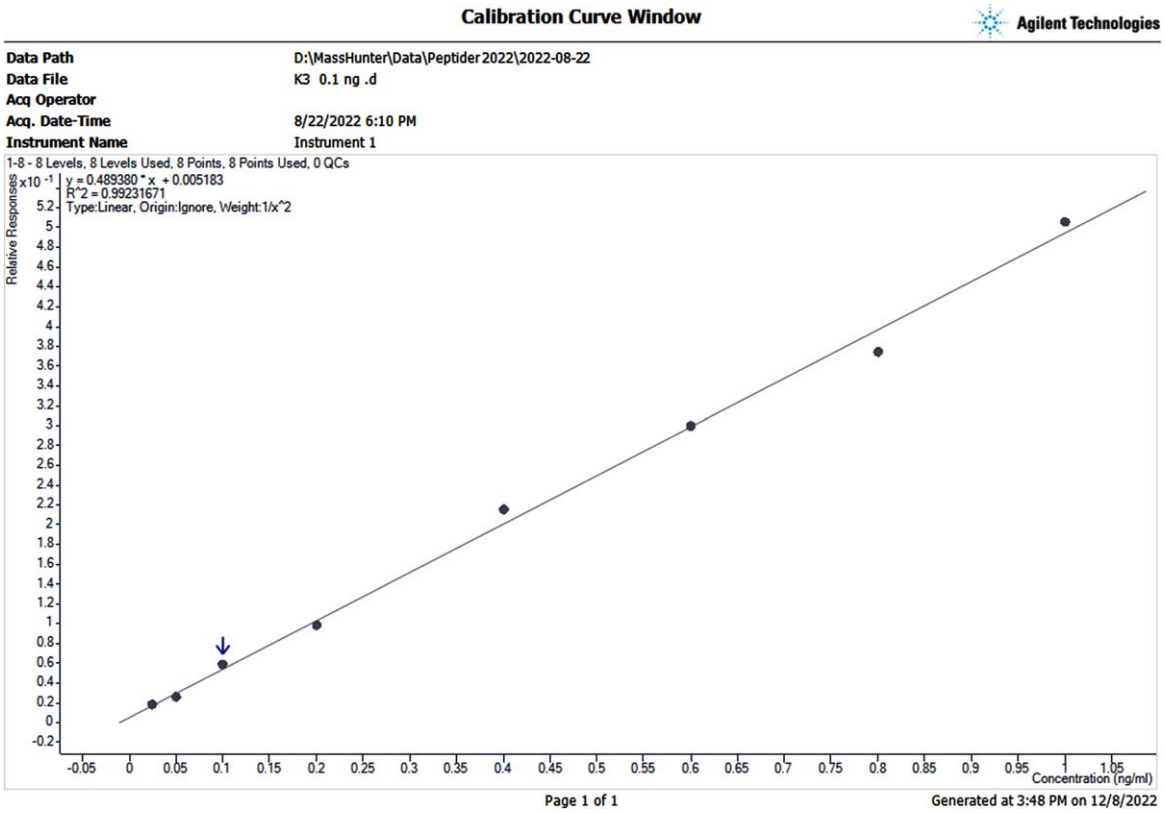

Fig. 3. Calibration curve for BCM-8 from 0,025 ng/mL to 1,00 ng/mL ($R^2 = 0,99$).

that this study should be followed by a larger and broader investigation. These future investigations should encompass the use of biomarkers and incorporate diet interventions. An extended study with several research groups involved and with a larger number of patients is planned.

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Author contributions

Conception: Tveiten D
Interpretation or analysis of data: Rønning PO, Tveiten D
Preparation of the manuscript: Tveiten D, Bryn V, Skjeldal OH
Revision for important intellectual content: Saugstad OD, Skjeldal OH, Isaksen J
Supervision: Skjeldal OH

Conflict of interest

Tveiten D is a co-owner and laboratory manager of Lab1, the company that performed the peptide analysis in urine for this project. Skjeldal OH is an Editorial Board Member of this journal, but was not involved in the peer-review process nor had access to any information regarding its peer-review. The other authors declare that they have no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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