

Huntington' disease – imbalance of amino acid levels in plasma of patients and mutation carriers

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Abstract

Determination of the plasma amino acid (AA) levels in Huntington's disease (HD) can make it possible to find the metabolic markers used in early diagnosis. The aim of the presented study was to determine the AA profile in plasma samples from HD patients and presymptomatic carriers, compared to healthy subjects. The AA profile was analyzed with HPLC. The study concerned 59 participants: 30 subjects with abnormal CAG repeats expansion (>36) in the *HTT* gene, and 29 healthy subjects. Each participant was analyzed with regard to the parameters characterizing the metabolic state and protein metabolism, such as: urea, creatinine, glucose, total protein, TSH (thyroid-stimulating hormone), cortisol, ESR (erythrocyte sedimentation rate), and CRP (C-reactive protein). Simple statistical comparisons showed 5 AA to be significantly lower in the HD group, compared to the control group, i.e.: Asn, His, Leu, Ser, Thr. Creatinine and creatinine clirens were found to be lower in the HD group, compared to controls, while ESR was noticed to be higher. As a result of Canonical Discriminant Analysis, 5 of all AA assayed (Leu, Gln, Asn, Ser and Lys) were selected as variables that allow distinguishing between HD patients and healthy subjects with 75% of correctness. Concerning AA profile and biochemical markers, Canonical Discriminant Analysis detected a panel of variables (Ser, Asn, Gln, Orn, Pro, Arg, Met, Cit, Val, TSH, glucose, urea, creatinine clirens, total protein, cortisol, CRP) distinguishing HD from the control group, with 90% of correctness. Among all the parameters tested, Asn and Ser were revealed in all statistical analyses and could be considered as potential plasma HD biomarkers.

Key words

Huntington's disease, biomarkers, amino acids, HPLC, plasma analysis, biochemical analysis.

List of abbreviations

AA – amino acid; **AQC** – 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; **BCAA** – branched chain amino acids; **CC** – calf circumference; **CDA** – Canonical Discriminant Analysis; **CG** – control group; **CRP** – C-reactive protein; **ESR** – erythrocyte sedimentation rate; **HDG** – HD group; **MNA** – Minimal Nutritional Assessment; **NMDA** – N-methyl-D-aspartate; **TFC** – Total Functional Capacity; **TSH** – thyroid-stimulating hormone; **UHDRS** – Unified Huntington Disease Rating Scale; **UHDRS TMS** – UHDRS Total Motor Score.

INTRODUCTION

Huntington's disease (HD) is a progressive, neurodegenerative, autosomal dominant disease. The responsible mutation is an abnormally expanded CAG trinucleotide repeat in the first exon of the *HTT* gene, encoding a polyglutamine stretch in the huntingtin protein. Transcriptional deregulation and altered energy metabolism have been implicated in HD pathogenesis [1]. Although transcriptional modifications, excitotoxicity, protein aggregation and loss of normal function of huntingtin, have been hypothesized to be responsible for the symptoms in patients, HD pathogenesis remains unclear. Unlike other neurodegenerative disorders, affected HD patients are known to lose weight, despite normal or even increased food intake [1].

The demonstration that mechanisms underlying weight loss in HD are related to disease onset would indicate that a

more systemic and possibly treatable defect may be implicated early in the pathophysiology of HD.

As shown by Mochel et al [1], a systemic metabolic defect involving BCAA in HD is associated with early weight loss. According to the authors, the role of BCAA in mitochondrial intermediary metabolism may indicate a systemic attempt at compensating for the early energy deficit in HD. The decrease in the levels of BCAA correlated with weight loss and with the UHDRS subscales scores and size of CAG repeats expansion in larger allele in HD patients [1].

OBJECTIVE

The aim of the presented study was to determine the profile of the AAs, including BCAA, in samples drawn from an HD group (HDG), compared to a control group (CG), using the HPLC method. Determination of the plasma AA levels in HDG can make the following possible:

– to find the metabolic markers that may be used in early diagnosis of HD;

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- to confirm the results obtained by Mochel et al [1], that lower levels of BCAA are correlated with weight loss and worse clinical status;
- to screen for other potential AA related biomarkers of HD.

This research can also assist with the proposal for a specially introduced diet supplementation enriched in the deficient AAs to reduce the effects of energy deficits in HD patients.

MATERIALS AND METHOD

Samples donors – HD patients, presymptomatic *HTT* mutation carriers and controls. All 59 participants were included in the study. Six presymptomatic carriers of mutation conditioning HD (5 females, one male) and 24 HD patients (12 females, 12 males) were included in one group labeled HDG (mean age: 45.5±10.0). 29 individuals free of HD were included in the control group, labeled CG (23 females and 6 males, mean age: 38±10.2). All presymptomatic *HTT* mutation carriers and patients were participants of the REGISTRY study [2] performed at University of Medical Sciences in Poznań, Poland. Informed consent was obtained from each subject or patient.

To exclude concomitant disorders disturbing metabolism an interview was conducted with each subject and a REGISTRY data-base search was performed. To exclude malnutrition, each participant was scored in MNA; fat tissue was measured using bioelectrical impedance analysis on TANITA Body Fat Monitor/Scale TBF-604, CC which reflects the condition of muscle mass, and BMI was calculated. To exclude inflammatory diseases, skin temperature was measured. A detailed biochemical profiles (obtained by blood analysis) for each participant were determined, especially the parameters known to reflect metabolic state and protein metabolism, (i.e. urea, creatinine, glucose, total protein, TSH, cortisol, ESR, and CRP). Neither changes in diet nor in conditions affecting body weight were recorded during the interviews with the patients. Among CG and HDG, the quantity of non-smokers to smokers was as follows: CG – 24 vs. 5; HDG – 21 vs. 9. Blood sampling was conducted under standardized conditions, identical for all participants and at the same time, i.e. at approx. 10:00. Food intake was prohibited after 16:00 the previous day till sampling time.

In the HDG, 4 subgroups were selected. The first group consisted of 6 presymptomatic individuals (as mentioned above), carriers of the *HTT* mutation, but without any significant signs of HD; CAG repeats in larger allele ranged from 38 – 47 (41±3). The next 3 subgroups included the symptomatic patients and were defined as:

I – III HD stages according to the TFC, i.e. I – 8 individuals (4 women, 4 men, TFC 13 – 11, CAG repeats 40–66 (46±9));

II – 12 individuals (7 women, 5 men, TFC 10 – 7, CAG repeats 42–81 (49±11));

III – 4 individuals (one woman, 3 men, TFC 6 – 3, CAG repeats 39–48 (42±4)).

Motor dysfunction in presymptomatic and HD affected individuals was evaluated with the UHDRS TMS.

Blood samples were collected into EDTA tubes, centrifuged to separate blood cells, and plasma samples stored at -70°C for further analysis.

HPLC analysis of amino acids profile. Chromatographic conditions have been defined according to Jaworska et al [3].

A HPLC Shimadzu apparatus able to form ternary gradient equipped with: two LC-10AT pumps and one LC-10AS pump, an SCL-10Avp control unit; autosampler SIL-10Dvp, degaser DGU-14A, fluorescence detector RF-10AXL, CLASS-VP software (version 5.3); column: AccQ-Tag 150x3.9mm; 4µm, Waters; precolumn: SecurityGard Cartridges C18 4.0×3.0mm, Phenomenex; column temperature: 30°C; fluorescence detector: ex=250nm; em=395nm; injection volume: 10µl; flow rate: 1 ml/min; mobile phase A: deionized water; mobile phase B: acetonitrile; mobile phase C: triethylamine acetate 50 mM, N,N-dimethyloctylamine 2 mM, pH = 5.10–5.13 [3].

Reference mixture was prepared using the final concentrations of AAs, similar to the physiological values in plasma [4].

Deproteinization procedure of plasma samples, as described by Jaworska et al [3], was performed with use of ultrafiltration process made with a Amicon Ultra 0.5 mL 10K Centrifugal Filters (Ultracel-10K membrane regenerated cellulose 10,000 MWCO). Deproteinized samples, as well as the AA reference, were subjected to derivatization procedure. Derivatization was proceeded at 55°C with a Waters AccQ-Fluor Reagent Kit containing: AccQ-Fluor Borate Buffer, AQC reagent and acetonitrile, according to manufacturer's protocol. The recovery for all AAs ranged from 90 – 104%.

The following reference standards were used for AAs analysis in the plasma samples: L-Arginine (as monohydrochloride), L-Isoleucine, L-Histidine (as monohydrochloride monohydrate), L-Leucine, L-Methionine, L-Lysine (as monohydrochloride), L-Phenylalanine, L-Threonine, L-Valine, L-Alanine, L-2-aminobutyric acid, L-Asparagine, L-Citrulline, L-Cystine, L-Glutamic Acid, L-Glutamine, Glycine, 4-L-Hydroxyproline, L-Proline, L-Serine, L-Ornithine (as monohydrochloride), L-Tyrosine and Taurine.

Statistical analysis. Statistics were performed using SYSTAT software ver. 13.00.05 for Windows (Systat Software Inc., Chicago, IL, USA). The data were expressed as median±quartile range as a consequence that the normality assumption was not obtained for all the variables. Non-parametric Whitney-Mann test was applied to verify the difference between groups. Simple linear correlation analysis was used to indicate correlations and strength of the relationship between variables. p-value <0.05 were considered statistically significant. Factor analysis and Canonical Discriminant analysis (CDA) were finally applied in order to select potential factors of diagnostic importance.

RESULTS

On the basis of Whitney-Mann test, 5 AA showed significant lower levels in HDG compared to CG (Tab. 1), although 2 of them with p<0.1. As shown in the biochemical profiles, lower levels in HDG as compared to CG were found for creatinine clirens (70.83±12.90 ml/min vs. 88.23±15.64 ml/min; p<0.05), but the HDG were characterized with higher values of ESR than controls (9±4 mm/h vs. 6±4; p<0.05). No significant differences in weight, BMI or CC between CG and HDG were found (61±8 kg vs. 67±9 kg, 22±3 vs. 23.0±3, and 36.0±3.3 vs. 36.0±4.0, respectively). Body temperature in all participants ranged within 36.5–37.4°C.

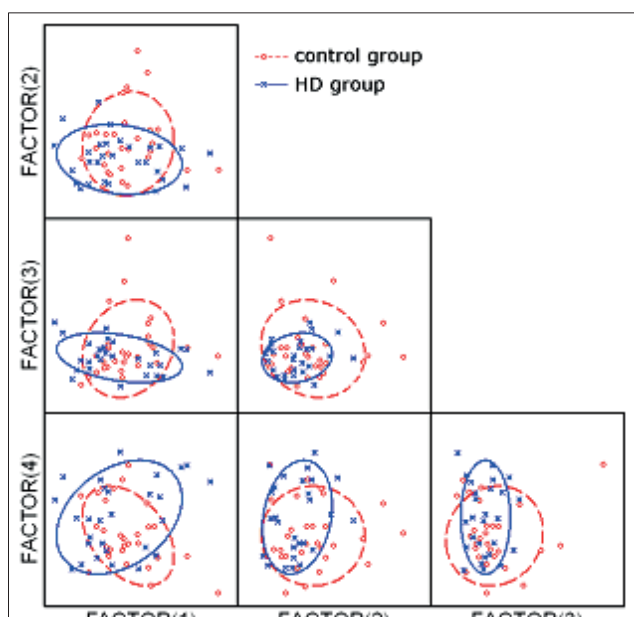
Table 1. Differences in AA levels in HDG, compared to CG selected on the basis of Whitney-Mann test [median±quartile range].

AA	CG [nM/ml]	HDG [nM/ml]	p-value
Asparagine	36.71±4.46	33.68±4.61	<0.05
Histidine	64.66±13.87	52.87±8.79	<0.05
Serine	96.25±11.91	88.93±11.85	<0.05
Leucine	88.82±18.52	84.49±18.16	<0.1
Threonine	120.71±28.62	103.29±20.74	<0.1

Normal plasma AA levels were of wide range and high inter- and intra-individual variability; therefore, even a relatively large difference in AA concentration could not be indicated as being statistically significant in the simple between-group comparisons, although it might be clinically important. For this reason, the AA profiles between HDG and CG were tested with advanced statistical analysis, such as factor analysis and discriminant analysis.

Factor analysis based on standardized data (*z-score* transformation) and using correlation matrix and principal components as the factor extraction method, was used to summarize data in a reduced number of factors. Appropriate rotation (varimax or quartimax) was applied to maximize distances between vectors. *Eigenvalues* and scree plot analysis revealed 4 factors (latent roots>2) explaining 55% of total variance. Factor 1 (20.2% of total variance) was related to the BCAA, sulphur AA and Lys; factor 2 (17.6% of total variance) was determined by: Asn, His, Ser, Arg, Gln, Hyp, Cit, Phe, Gly and Thr. Factors 3 and 4 (8.5% and 7.8% of total variance, respectively) were associated with creatinine clirens, CRP and ESR levels. However, the scatterplot matrix of factor scores calculated for CG and HDG did not show significant data segregation (Fig. 1).

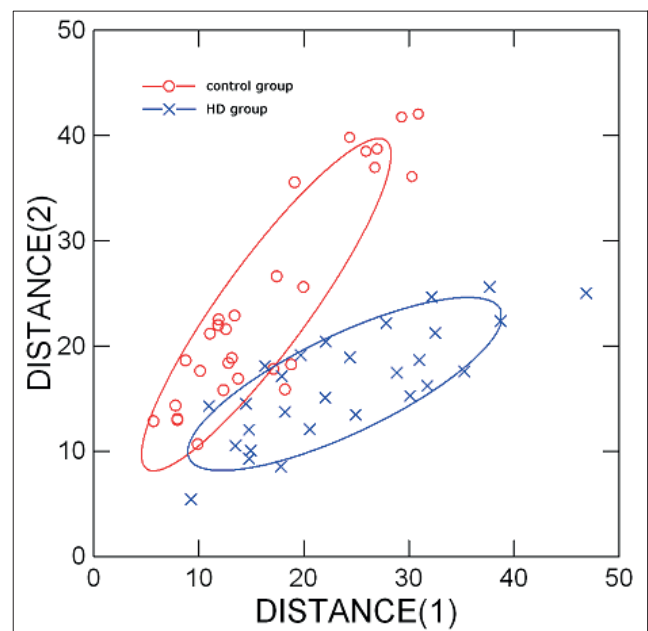
CDA was based on the extraction of linear discriminant functions of the independent variables applying backward stepwise estimation. All variables of biochemical parameters

**Figure 1.** Matrix scatterplot of factor scores calculated for HDG and CG presenting an overview of data segregation. Confidence ellipses present 1 SD of the sample means of the x and y variables.

and AAs were initially included into an estimated model, and subsequently stepwise removed based on the lowest significance with α -to remove set at 0.15 and tolerance at 0.001. Finally, the discriminant function was developed based on 17 variables according to decreasing classification power (Tab. 2). The function was able to discriminate between the patients and the controls with correctness of ca. 90% (Fig. 2).

Table 2. List of parameters and coefficients of discriminant function that allows to distinguish HDG and CG with ca. 90% of correctness.

Canonical Discriminant Function	
Variable	Function coefficient
Creatinine clirens	1.177
CRP	1.214
Serine	1.197
Asparagine	1.226
Cortisol	-0.767
Glutamine	-0.814
Ornithine	1.13
Proline	0.697
Arginine	-1.014
Protein	0.738
Creatinine	0.725
Urea	0.673
Glutamic acid	-0.596
Methionine	-0.994
TSH	-0.591
Citrulline	0.617
Valine	-0.618
constant	-0.094

**Figure 2.** Mahalanobis Distance-Square plot presents the ability to distinguish HDG and CG based on variables listed in Table 2.

The same analysis was performed with variable-set limited to AA profile. As a result of CDA, 5 amino acids were selected: Ile, Gln, Asn, Ser and Lys and Canonical Discriminant

function parameters according to decreasing classification power (Tab. 2). The function was able to discriminate between the patients and the controls with correctness of ca.75% (Fig. 3).

Table 3. List of AA and coefficients of discriminant function that allows to distinguish HDG and CG with ca. 75% of correctness.

Canonical Discriminant Function	
Variable	Function coefficient
Isoleucine	1.185
Glutamine	-0.77
Asparagine	0.718
Serine	0.689
Lysine	-0.988
constant	0

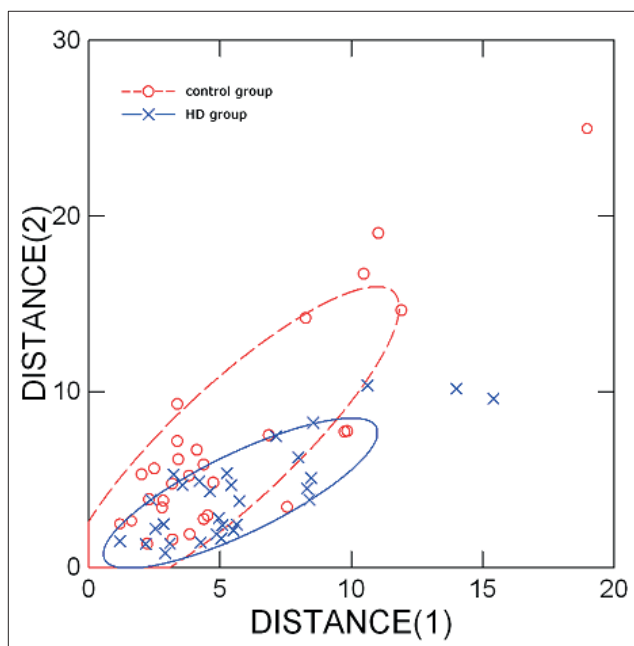


Figure 3. Mahalanobis Distance-Square plot presents the ability to distinguish HDG and CG based on determination of AAs listed in Table 3.

The given discrimination between CG and HDG showed no relation with gender (Fig. 4). Significant correlations between UHDRS and neither AA nor biochemical parameters were observed (data not shown). The HD stage could not be taken into account in the analysis due to small number of the subjects in each HD stage.

DISCUSSION AND CONCLUSIONS

The presented study did not confirm weight loss as a potential feature related to HD, as considered, among others, by Mochel et al [1] and van der Burg et al [5]. However, among the parameters discriminating CG and HDG at 90%, cortisol, glucose, TSH, total protein, creatinine clirens, and CRP could be found, which point to some metabolic abnormalities as a potential biomarker of HD, although the values determined for these parameters ranged references in each participant. Weir et al [6] mentioned that the increased plasma levels of cortisol and CRP prove increased anxiety, stress and

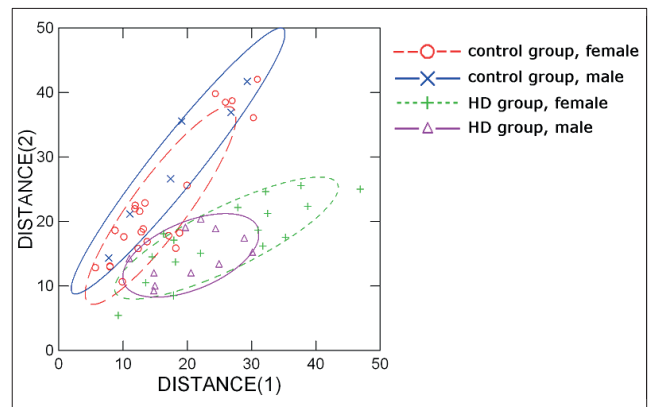


Figure 4. Mahalanobis Distance-Square plot (based on parameters listed in Table 2) shows that recognition between CG and HDG is regardless of gender.

depression – the symptoms reported in HD, as well as indicating an inflammatory component in the pathogenesis of the disease. Stoy et al [7] proved persistent inflammation in HD, noting the elevated baseline level of CRP in the blood of the patients.

On the basis of the presented study, it cannot be confirmed that BCAA are plasma HD biomarkers, in the way suggested by Mochel et al [1, 8], although BCAA – both individually or as a group – were found as variables differentiating HDG and CG, i.e. Ile was selected in CDA, or Factor1 was determined by BCAA in the factor analysis. The majority of AAs revealed in the current statistical analysis have already been mentioned by other authors as potentially related to HD, or to other neurodegenerative diseases [9, 10, 11]. Due to the small number of subjects in the presented study, it was not possible to draw strictly defined conclusions about the precise role of AAs distinguished in the statistical analysis in pathogenesis of HD. Besides, the interference between AA levels and some medicines used by the participants cannot be excluded.

The AA revealed in Whitney-Mann test and CDA, and which could be considered as plausible plasma biomarkers, are Asn and Ser. A significant decrease ($p < 0.001$) of Asn in CSF of HDG, compared to the non-choreic control patients, was noted by Oepen et al [11]. The presented results also indicate a significant lower level of AA in HDG ($p < 0.05$). The role of Asn – non essential AA – in HD pathogenesis may be related to the metabolism of Gln, i.e. the formation of excitotoxic Glu catalysed by asparagine synthase, as well as to the metabolism of excitotoxic Asp which is transformed into Asn in the same reaction which, besides an N-donor, also requires ATP. Thus, the level of Asn may be the marker of the Asp level which, in turn, through formation of oxaloacetate, participates in cell energy metabolism. The next product of Asp transformation is N-acetylaspartate which favours conversion of Glu into α -ketoglutarate, or as itself may be converted to oxaloacetate. Both enter into the Krebs cycle to produce energy. N-acetylaspartate is involved in facilitating energy metabolism in neuronal mitochondria with probably preferential use the aspartate aminotransferase, although a direct connection between N-acetylaspartate synthesis and ATP is still not clear. Decrease in the N-acetylaspartate level in the mouse model for HD has been well-documented and coincided with the onset of symptoms [12, 13]. Isomerization of Asp and deamidation of Asn give rise to L-isoaspartate. In turn, isoaspartates have been identified as pathologically-deposited proteins in neurodegenerative brain tissue [14].

Ser is also a non-essential AA. It is a key for the synthesis of the neurotransmitters Gly and D-serine in the brain. In the Central Nervous System, these AAs were shown to be especially important by acting as co-agonists in the regulation of NMDA receptor function [15]. Reilmann et al [9] noted no significant change in Ser level in HD plasma. In the presented study, the Ser level was lower in HDG, compared to CG ($p < 0.05$). Ser acts in biological processes in a way that can be directly or indirectly related to HD pathogenesis, i.e. it modulates Glu receptors which have important neurologic functions. In turn, Glu influences neuronal survival [16]. As shown by Sakagami et al [17], Ser depletion in HL-60 cells accumulated the G1 arrested cells and produced increasing numbers of the apoptotic cells. Supplementation of Ser significantly extended the period of logarithmic cell growth.

The decreased plasma Asn and Ser levels in HDG have been distinguished among other AAs as the most plausible parameters characterizing this group, and prove the role of activation of NMDA receptors as well as energy metabolism in HD pathogenesis. Both AAs are non-essential, thus their levels do not directly reflect dietary habits.

Although at this stage of the research, mainly due to the small number of subjects, it is difficult to be precise about the interdependence of the parameters revealed by CDA. However, the results of statistical analysis indicate the direction for further work in the search for HD plasma biomarkers.

This study includes preliminary studies prompting the set of parameters analyzed in the plasma samples which may potentially discriminate controls and HD patients. Further research is needed with a bigger number of subjects to unequivocally prove the results obtained, and to define the plasma biomarkers among the parameters tested.

Ethical statement

This study was approved on 3 September 2009 by the Bioethical Committee at the University of Medical Science in Poznań, Poland, Agreement No. 770/09. All studies on humans were by the Agreement and performed in accordance with Good Clinical Practice laid down in the 1964 Helsinki Declaration. All persons gave their informed consent prior to their inclusion in the study.

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