BBA - Molecular Basis of Disease xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Targeted biochemical profiling of brain from Huntington's disease patients reveals novel metabolic pathways of interest

Stewart F. Graham[a,b,*], Xiaobei Pan[c], Ali Yilmaz[a], Shirin Macias[c], Andrew Robinson[d], David Mann[d], Brian D. Green[c]

[a] Beaumont Health, 3811 W. 13 Mile Road, Royal Oak, MI 48073, United States
[b] Oakland University-William Beaumont School of Medicine, Rochester, MI 48309, United States
[c] Advanced Asset Technology Centre, Institute for Global Food Security, Queen's University Belfast, Belfast, UK
[d] Institute of Brain Behavior and Mental Health, University of Manchester, UK

ARTICLE INFO

Keywords:
Huntington's disease
Metabolomics
Brain
Biochemical pathways
Biomarkers

ABSTRACT

Huntington's disease (HD) is a devastating, progressive neurodegenerative disease with a distinct phenotype characterized by chorea and dystonia, incoordination, cognitive decline and behavioral difficulties. The precise mechanisms of HD progression are poorly understood; however, it is known that there is an expansion of the trinucleotide cytosine-adenine-guanine (CAG) repeat in the *Huntingtin* gene. Herein DI/LC-MS/MS was used to accurately identify and quantify 185 metabolites in post mortem frontal lobe and striatum from HD patients and healthy control cases. The findings link changes in energy metabolism and phospholipid metabolism to HD pathology and also demonstrate significant reductions in neurotransmitters. Further investigation into the oxidation of fatty acids and phospholipid metabolism in pre-clinical models of HD are clearly warranted for the identification of potential therapies. Additionally, panels of 5 metabolite biomarkers were identified in both the frontal lobe (AUC = 0.962 (95% CI: 0.85–1.00) and striatum (AUC = 0.988 (95% CI: 0.899–1.00). This could have clinical utility in more accessible biomatrices such as blood serum for the early detection of those entering the prodromal phase of the disease, when treatment is believed to be most effective. Further evaluation of these biomarker panels in human cohorts is justified to determine their clinical efficacy.

1. Introduction

Huntington's disease (HD) is an autosomal-dominant, progressive neurodegenerative disease. Its distinct phenotype is characterized by chorea and dystonia, incoordination, cognitive decline and behavioral difficulties [1]. It is known that in HD there is an expansion of the trinucleotide cytosine-adenine-guanine (CAG) repeat in the first exon of the *Huntingtin* (HTT) gene [2]. Repeat expansions of 36+ can cause the disease with CAG repeat length being inversely related to age of onset (median age 40) [3], for example expansions of 60–70 lead to the development of a more aggressive form of the disease, leading to juvenile onset [4]. Despite this, the precise pathophysiological mechanisms of HD progression, including its effect on metabolism are poorly understood. The prevalence of HD is significantly higher in Western Europe, North America and Australia compared with Asian populations but the disease is still estimated to affect 5–10 people per 100,000 population worldwide [5,6].

Metabolomics is a relatively new discipline which involves the comprehensive and simultaneous measurement of chemical processes involving metabolites, holding huge potential for the discovery of biomarkers of disease and for providing insight into disease pathogenesis [7]. One particular advantage it holds over other technologies such as genomics, transcriptomics and proteomics is that it directly assesses the metabolic changes of an organism providing a global molecular representation of its phenotype [8]. Metabolomics has shed some light on the metabolic milieu occurring as a consequence of HD pathology. A number of groups undertaking metabolomic profiling have found evidence of metabolites such as amino acids, lipids and

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; aa, acyl; ae, diacyl; ATP, adenosine triphosphate; AUC, area under the curve; CAG, trinucleotide cytosine-adenine-guanine; DI/LC-MS/MS, direct injection liquid chromatography mass spectrometry; FDR, false discovery date; HD, Huntington's disease; HMDB, Human Metabolome Database; HRMS, high resolution mass spectrometry; HTT, Huntingtin gene; LASSO, Least Absolute Shrinkage and Selection Operator; LOOCV, leave one out cross validation; MSEA, metabolite set enrichment analysis; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; PM, post-mortem; ROC, receiver operated characteristic; SVD, small vessel disease; VIP, variable importance in projection

* Corresponding author at: Oakland University-William Beaumont School of Medicine, Rochester, MI 48309, United States.
E-mail address: stewart.graham@beaumont.edu (S.F. Graham).

https://doi.org/10.1016/j.bbadis.2018.04.012
Received 13 February 2018; Received in revised form 28 March 2018; Accepted 17 April 2018

neurotransmitters being significantly altered in HD [9–16].

The overriding aims of all the above studies is the same, i.e. to employ metabolomics to improve HD diagnosis which could in turn advance study design, clinical trials and clinical care through improved prognosis and earlier intervention [17]. The aim of the present study was to undertake metabolomic profiling of HD striatum and frontal lobe tissue, but importantly we used a readily available tandem mass spectrometry-based kit which identified and quantified 185 metabolites in PM brain. These two specific regions were chosen as both are significantly affected in the brain of those suffering from HD. Striatum is commonly affected due to neurodegeneration and cell loss [18] whereas significant atrophy (associated with some of the cognitive impairment and clinical decline observed in patients with HD) has been reported in the frontal lobe [19]. The main goals were to discover novel central nervous system biomarkers of HD and to gain a more comprehensive understanding of the metabolic and pathophysiological processes involved in HD.

2. Materials and methods

2.1. Post mortem brain samples

Brain tissue was obtained from the University of Manchester Brain and Tissue Bank as previously described [9,10]. Newcastle & North Tyneside 1 Research Ethics Committee (REC Reference# 09/H0906/52 + 5) approved the use of the PM brain samples described herein. In addition, this study was approved by the Beaumont Institutional Review Board (IRB# 2014–353). Briefly, brain tissue (frontal lobe and striatum) was obtained from post-mortem HD cases (n = 14) and from control subjects (n = 14) with no evident Huntington's pathology. All HD cases demonstrated a moderate to severe atrophied corpus striatum consistent with grades 2 or 3. Exact CAG repeat numbers were not available however the diagnosis was confirmed using genetic testing except for 2 cases, BBN_3211 and BBN_6070. Diagnosis of HD for these two cases was made by the presence of ubiquitinated/p62 positive intra-nuclear inclusions within cortical and striatal neurons (all other HD brains also demonstrated such inclusions). These inclusions were not observed in the control cases. A summary of the demographic information is available as Table 1. A more comprehensive summary is available as Supplementary Table 1 to include Vonsattel grading information, PM delays and CAG repeat length where available.

2.2. Sample preparation and DI/LC-MS/MS

Post-mortem brain samples were prepared and analyzed using a method previously reported by Urban et al., [20] and our group [7,21]. Post-mortem brain samples were dried under vacuum using a Christ Alpha 1-4LD Plus freeze dryer (IMA Life) and milled to a fine powdered under liquid nitrogen (to reduce any potential proteolysis) using a 6870-freezer mill (SpexSamplePrep). Of the milled, lyophilized tissue, 10 mg was extracted in 300 µl of 85:15 ethanol:phosphate buffered saline solution (pH 7.4). After 5 min of sonication the samples were mixed for 30 s and centrifuged at 10,000*g* at 4 °C for 5 min and the supernatant collected. 10 µl of the brain extract was analyzed using the targeted, commercially available quantitative DI/LC-MS/MS AbsoluteIDQ p180 kit (Biocrates, Innsbruck, Austria) as previously described by our group [7,22,23] and all sample preparatory steps were completed as detailed by the manufacturer. Metabolites (amino acids and biogenic amines) were separated based on retention time using a reverse phase column (Waters ACQUITY UPLC BEH C18 2.1 × 50 mm, 1.7 µm; Wexford, Ireland) coupled to a UPLC (I-Class, Waters Corporation) and quantified using a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Corporation) operating in the multiple reaction monitoring mode. All remaining compounds (acylcarnitines, hexoses, glycerophospholipids, and sphingolipids) were quantified using the same mass spectrometer using flow injection analysis, operating in multiple reaction monitoring mode. Metabolite concentrations were calculated and expressed in micromolar (µM).

Table 1
Demographic characteristics of the study samples.

	Total sample, N = 28	Controls, N = 14	HD, N = 14
Age, Mean (SD)	68 (17.20)	78.5 (13.46)	54.64 (12.39)
Sex, n (% female)	11 (39.28)	6 (42.85)	6 (42.85)
PM delay (hours), Mean (SD)	68.57 (58.57)	77.36 (74.34)	69.29 (39.53)
Brain Weights (g), Mean (SD)	1231.94 (184.39)	1268.89 (186.22)	1198.7 (184.15)

2.3. Statistical analysis

Standard metabolomics statistical methods were used as previously reported by our group and other experts in the field [22,24,25]. All data were analyzed using MetaboAnalyst (v3.0) [26–28] and univariate analyses consisted of a Student's *t*-test for metabolites exhibiting a normal distribution or the Wilcoxon Mann-Whitney test for metabolites exhibiting non-normal distributions. False discovery rates (FDR, q-value) were calculated to account for multiple comparisons. All data were normalized to the median and auto-scaled prior to multivariate analysis. Principal component analysis (PCA) was used to identify any potential outliers followed by partial least squares-discriminant analysis (PLS-DA) to highlight significant metabolites which explain the maximum amount of variation between the groups. Significance of the PLS-DA models was assessed using the leave-one-out cross-validation (LOOCV) technique. Models with a p-value < 0.05 were considered significant. Variable importance in projection (VIP) plots was created to identify the top 15 metabolites responsible for the observed separation between groups.

Receiver operating characteristic (ROC) curves were developed to generate the greatest predictive models for each group classification and metabolites were chosen for each model as chosen from the Variable Importance in Projection (VIP) scores or using the Least Absolute Shrinkage and Selection Operator (LASSO). Models were validated using 100-fold cross validations and permutation testing (1000 iterations). Models with a p-value < 0.05 were considered significant.

2.4. Metabolite set enrichment analysis

Using MetaboAnalyst (v3.0) we completed the metabolite set enrichment analysis (MSEA) as previously described by our group [10,21–23,29]. All data were normalized to the median and auto-scaled. The *Homo sapiens* pathway library was selected and all compounds in that particular pathway library were used when referencing the metabolome. We employed Fisher's exact test for the over-representation analysis and we chose “relative betweenness centrality” pathway topology testing. Pathways that had both a Holm adjusted p-value < 0.05 and q-value < 0.3 were considered significantly perturbed due to HD.

3. Results

To biochemically profile the frontal lobe and striatum from HD patients we employed DI/LC-MS/MS in combination with the commercially available metabolomics kit (Biocrates, Innsbruck, Austria). In total, we accurately identified and quantified 185 metabolites to include acylcarnitines, amino acids, glycerophospholipids, hexoses, sphingolipids and biogenic amines. Of the limited available demographic information, only age was found to be statistically significantly different between cases and controls ($p = 0.0001$). The results of the univariate analysis for the metabolites recorded in the frontal lobe of