Jardim Laura Bannach (Orcid ID: 0000-0001-6907-5068)
Costa Maria do Carmo (Orcid ID: 0000-0001-8466-4864)

Blood *DDIT4* and *TRIM13* transcript levels mark the early stages of Machado-Joseph disease.

Ana F. Ferreira, PhD[1,2*,] Mafalda Raposo, PhD[3], Emily D. Shaw, MSc[4], Louisa Liu[4], João Vasconcelos, MD[5], Teresa Kay, MD[6], Conceição Bettencourt, PhD[7], Maria Luiza Saraiva-Pereira, MD, PhD[8,9], Laura Bannach Jardim, MD, PhD[9,10], Maria do Carmo Costa, PhD[4*†], Manuela Lima, PhD[1,2*†]

1. Faculdade de Ciências e Tecnologia, Universidade dos Açores, 9500-321 Ponta Delgada, Portugal.

2. UMIB - Unit for Multidisciplinary Research in Biomedicine, ICBAS - School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal

3. Instituto de Biologia Molecular e Celular (IBMC), Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, 4200-135 Porto, Portugal.

4. Department of Neurology, Michigan Medicine, University of Michigan, 48109-2200 Michigan, USA.

5. Serviço de Neurologia, Hospital do Divino Espírito Santo (HDES), 9500-370 Ponta Delgada, Açores, Portugal.

6. Serviço de Genética Clínica, Hospital Dona Estefânia, 1169-045 Lisboa, Portugal.

7. Department of Neurodegenerative Disease and Queen Square Brain Bank for Neurological Disorders, UCL Queen Square Institute of Neurology, WC1N 1PJ London.

8. Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, 90035-003 Rio Grande do Sul, Brazil.

9. Serviço de Genética Médica/Centros de Pesquisa Clínica e Experimental, Hospital de Clínicas de Porto Alegre, 90035-903 Porto Alegre, Brazil.

10. Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul, 90035-002, Porto Alegre, Brazil.

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ana.27222

This article is protected by copyright. All rights reserved.

Author Manuscript

†These senior authors contributed equally to this work.

*Corresponding authors:

Ana F. Ferreira, Ph.D.

Faculdade de Ciências e Tecnologia, Universidade dos Açores

Rua Mãe de Deus, N.13

9500-321 Ponta Delgada, Portugal

Phone: +351 296 650 000

E-mail address: ana.fb.ferreira@uac.pt

Maria do Carmo Costa, Ph.D.

Department of Neurology, University of Michigan

109 Zina Pitcher Place

A. Alfred Taubman Biomedical Sciences Research Building, Room 4027

Ann Arbor, MI 48109-2200, USA

Phone: +1 734-764-4094

E-mail address: mariadoc@med.umich.edu

Manuela Lima, Ph.D.

Faculdade de Ciências e Tecnologia, Universidade dos Açores

Rua Mãe de Deus, 13

9500-321 Ponta Delgada, Portugal

Phone: +351 296 650 118

E-mail address: maria.mm.lima@uac.pt

Author Manuscript

Running head: Blood *DDIT4* and *TRIM13* transcripts are MJD markers

Number of characters in the title: 88 characters with spaces

Number of characters in the running head: 50 characters with spaces

Number of words in the abstract: 244 words

Number of words in the Introduction: 430 words

Number of words in the discussion: 1400 words

Number of words in the body of the manuscript: 4500 words

Number of figures, color figures and tables: 6 figures (black and white)

Number of supplementary figures and supplementary tables: 1 supplementary figure, 7 supplementary tables

Author Manuscript

Summary for Social Media If Published, and provide the information below:

1. If you and/or a co-author has a Twitter handle that you would like to be tagged, please enter it here. (format: @AUTHORSHANDLE). No co-author has a Twitter.

2. What is the current knowledge on the topic? While a few serum/plasma and blood transcriptional biomarkers are known for Machado-Joseph disease (MJD), additional blood transcriptional changes that can trace MJD clinical stages with high accuracy remain to be unveiled.

3. What question did this study address? This study aimed to identify transcriptional changes in blood samples from individuals with MJD as biomarkers of specific disease stages.

4. What does this study add to our knowledge? This study identifies the high potential of using *DDIT4* and *TRIM13* transcript levels as blood-based biomarkers of MJD. Importantly, combined blood abundance of *DDIT4* and *TRIM13* transcripts distinguishes MJD subjects in preclinical and early symptomatic stages from healthy individuals with 90% or higher accuracy. Interestingly, levels of TRIM13 protein are altered in the cerebral cortex of MJD brains.

5. How might this potentially impact on the practice of neurology? Non-invasive assessment of blood abundance of *DDIT4* and *TRIM13* transcripts may be used alone or in combination with other biomarkers to distinguish MJD subjects from healthy individuals enrolled in clinical trials.

Author Manuscript

Author Manuscript

Abstract

Objective: Abundance of select transcripts and proteins has been found to be dysregulated in blood samples of Machado-Joseph disease (MJD) carriers. Here, we aimed to: 1) identify blood transcriptional changes as potential biomarkers of MJD; 2) correlate levels of differentially expressed blood transcripts with MJD carriers features; and 3) evaluate whether the identified differential abundance of blood transcripts in MJD subjects is preserved in MJD brains.

Methods: We used unbiased RNA microarray and quantitative PCR to assess transcript levels in blood and brain samples, and Western blot analysis to evaluate the abundance of specific proteins in brain samples.

Results: We observed consistent dysregulation of *DDIT4*, *TRIM13* and *P2RY13* transcriptional levels in blood of MJD subjects from preclinical to symptomatic stages in Azorean and Brazilian cohorts. Combined blood *DDIT4* and *TRIM13* transcriptional levels displays a very high accuracy to discriminate MJD carriers from matched controls (AUC≥0.90). Levels of *P2RY13* transcripts correlate with age at onset, and abundance of *DDIT4* and *TRIM13* transcripts correlate with the expanded CAG repeat size in combined Azorean and Brazilian patients; and levels of *TRIM13* transcripts correlate with age at onset of early-stage Azorean patients. Moreover, the abundance of TRIM13 protein is increased in the cerebral cortex of MJD patients.

Interpretation: Overall, blood *DDIT4* and *TRIM13* transcript levels are potential biomarkers of MJD. Cellular processes involving *DDIT4*, *TRIM13* and *P2RY13* appear to be commonly dysregulated in blood and brain of MJD subjects indicating the involvement of these genes in MJD pathogenesis.

Keywords: polyglutamine disease; spinocerebellar ataxia type 3; ATXN3; transcriptional dysregulation; molecular biomarkers; neurodegeneration

Author Manuscript

Introduction

Machado-Joseph disease, also known as Spinocerebellar Ataxia Type 3, is a slowly progressive dominant ataxia of late onset [1] predominantly affecting the cerebellar, pyramidal, extrapyramidal, motor neuron and oculomotor systems [2,3]. The MJD-causative *ATXN3* gene contains a polyglutamine (polyQ)-encoding CAG tract that in non-mutated chromosomes ranges from 12 to 44 repeats, and consensually surpasses 60 repeats in mutated chromosomes [4]. The overt disease is preceded by a preclinical phase in which MJD mutation carriers show brain structural changes [5,6] and may manifest nystagmus [7,8]. *ATXN3* encodes ataxin-3 (ATXN3), a deubiquitinating enzyme that is ubiquitously expressed in various cell types in the central nervous system and in peripheral tissues [9]. Evidence shows that mutated ATXN3 triggers a cascade of pathogenic events leading to several cellular alterations including transcriptional dysregulation [9,10]. In fact, global changes in gene expression have been identified in peripheral blood samples from MJD carriers [11–15], including preclinical subjects [13,14]. Therefore, tracking significant transcriptional alterations in blood samples of MJD subjects should: i) provide new insights about the peripheral molecular dysfunction in MJD, and ii) allow the identification of novel minimally invasive molecular biomarkers of MJD, particularly in preclinical stages where clinical scales are devoid of utility. Indeed, several studies identified changes in levels of transcripts and proteins in peripheral blood samples of MJD carriers, such as increase of the pro-inflammatory factor eotaxin [16], decrease of antioxidant capability and excessive production of reactive oxygen species [17], accumulation of a common mitochondrial DNA deletion (m.8482_13460del4977) [18], reduced *BCL2* transcript levels [15], and reduced *BCL2*/*BAX* transcript ratio [18].

Because clinical trials enrolling MJD subjects at preclinical and early disease stages are expected to demonstrate the maximal efficacy of disease-modifying therapeutic agents [19], identification of biomarkers of these early disease stages that can be assessed across clinical trials are an upmost need [20]. Increased blood levels of the general biomarker of neurodegeneration -

Author Manuscript

neurofilament light chain (NfL) - and of mutant ATXN3 are currently the most promising blood biomarkers for MJD. However, further studies are still needed to establish their value as progression, proximity-to-onset, and putative treatment-response markers and thus identification of blood MJD biomarkers is still an unmet need.

Hence, in this study, we sought to identify transcriptional changes in peripheral blood of MJD subjects that could be molecular biomarkers of MJD from preclinical to symptomatic phases and, ideally, of disease progression. To investigate the behavior of blood-based transcriptional changes in the primarily affected tissue in MJD, we further assessed the expression of promising candidates at the transcript and protein levels in post-mortem brain samples of MJD patients and controls.

Subjects and methods

Blood samples and clinical data

Approval by local Ethics Committees - all participants provided written informed consent. This study included a total of 83 blood samples from MJD patients - 59 patients representing several stages of the disease (39 from the Azorean cohort and 20 from the Brazilian cohort) and 24 additional Azorean patients in an early symptomatic stage of disease (EP) (five or less years of disease duration). Overall, the cohort of Azorean EP patients included 12 patients from the mixed disease stages group and an additional 24 EP patients (n=36). The five year cut-off used to define EP status was based on MJD natural history data that showed very slow disease progression (gait impairment) during the first five years of disease [21]. All patients in each cohort underwent a neurological evaluation by a single experienced neurologist in MJD (LBJ in Brazil and JV in Azores). In a subset of patients the score of the Neurological Examination Score for Spinocerebellar Ataxia [22] was also assessed. Age at onset was defined as the age of appearance of the first symptoms (gait ataxia and/or diplopia) reported by the patient or a close relative.

Author Manuscript

Disease duration was defined as the time elapsed between age at onset and age at neurological evaluation during which blood was collected. After being molecularly confirmed as carriers of the *ATXN3* mutation (Azorean Genetic Counseling and Predictive Test Program, Regional Health System, Azores), 19 Azorean preclinical subjects (PC) were also enrolled in the study. For preclinical subjects, the number of years between the age at neurological evaluation/blood collection and the predicted age at disease onset (years to onset) was calculated as previously described [23]. Sizing of the *ATXN3* CAG repeat was performed as previously reported [24]. Additionally, blood samples from 98 healthy controls, molecularly excluded for the *ATXN3* mutation [24], from the two populational origins (Azores (n=78), Brazil (n=20)) were included in this study. Demographic, genetic and clinical information of all individuals used in this study is shown in Supplementary Table 1.

Human brain samples

Approvals: IBCA00000265; Parecer 26/2018. Post-mortem frozen brain samples from two brain regions severely affected in MJD (dentate cerebellar nucleus (DCN) and pons) [25], and from a region minimally affected by the disease (cerebral cortex) [26,27] of deidentified molecularly confirmed MJD patients (n=5) and controls (n=9) (University of Michigan Brain Bank) were used in this study. The CAG repeat number of post-mortem brain samples was determined at Laragen Inc. as previously described [28]. Demographic, genetic, and clinical information of individuals from whom brain samples were used here is shown in Supplementary Tables 1 and 2.

Experimental design

The experimental workflow used here is displayed in Figure 1. Briefly, 28 peripheral blood RNA samples from the Azorean cohort (group #1) were used to perform a gene expression microarray (Figure 1A). To identify dysregulated genes in MJD subjects comparing with controls and to detect differences between the preclinical and the symptomatic stages of the disease, gene

Author Manuscript

expression levels were compared pairwise between the three groups (PC vs. controls, patients vs. controls, and PC vs. patients). Among the dysregulated genes in blood (Benjamini-Hochberg false discovery rate adjusted *p*-value (*q*-value) lower than 0.05), 24 genes known to be expressed in the human brain [29] and without copy number variation [30] were selected for validation by quantitative real-time PCR (qPCR) in the same samples used in the microarray (group #1, Figure 1B). After technical validation, a set of genes that maintained the same direction of effect (in at least two comparisons) as in the microarray was selected if genes displayed significant differences ($p<0.05$) (n=8), or if belonged to a family of genes previously observed to be dysregulated in polyQ diseases (n=4) [31–37]. Expression levels of the selected genes (n=12) were then assessed in independent Azorean blood samples from group #2 (Figure 1C.1). Next, transcript levels of five of these 12 genes were evaluated in patients and controls from a Brazilian cohort (group #3) (Figure 1C.2). Blood *P2RY13* transcript levels, priorly identified by us as a potential biomarker of MJD [11], were also analyzed in the Brazilian cohort (Figure 1C.2). Expression levels of the three genes showing consistent dysregulation in blood samples of Azorean and Brazilian patients compared to controls were further evaluated in RNA from blood samples of: i) MJD Azorean subjects at preclinical stages of disease (PC subjects from groups 1 and 2 (n=19); note that one preclinical subject was replaced by another subject due to the lack of RNA sample) and at early stages of disease (including 12 patients from the mixed disease stages from groups 1 and 2, plus 24 additional EP patients); ii) age- and sex-matched paired controls (group #4) (Figure 1D) . The expression of these three genes was also assessed at the transcriptional and protein levels in brain tissue from MJD patients and controls (Figure 1E).

RNA extraction and cDNA synthesis

Total RNA was extracted from peripheral blood samples of MJD carriers and controls as previously described [11]. Total blood cDNA was generated from 0.5µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Total RNA extracted from three

brain regions of MJD patients and controls was extracted using Trizol (Invitrogen) and purified using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Brain RNA concentration and integrity were assessed using the Agilent 2100 Bioanalyzer (Supplementary Table 2). Total brain cDNA was generated from 1.25µg of total RNA using the iScript cDNA synthesis kit (BIORAD).

Microarray analysis

Microarray analysis was conducted to identify transcriptional alterations in peripheral blood samples across the three biological comparison groups: PC vs. controls, patients vs. controls, PC vs. patients (Figure 1A; Supplementary Table 1). RNA was amplified, biotin-labeled and directly hybridized to the Illumina HumanHT-12 v4.0 Expression BeadChip (47,231 probes) (Illumina). Slides were scanned using the Illumina iScan and the signal was extracted and normalized using the GenomeStudio software. All array procedures were conducted at the UCL Genomics facility. Microarray probes related to mRNAs encoding unmapped genes, non-coding genes, pseudogenes and open-reading frames were excluded from further analysis. Mayday 2.9 software [38] was used to compare the gene expression levels between the three comparison groups (q<0.05). Microarray data for the 24 genes selected for qPCR validations are shown in Supplementary Table 3.

Quantitative PCR

qPCR experiments (Figure 1B-E; Supplementary Table 1) were conducted using the TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Supplementary Table 4), on a StepOnePlus Real-Time PCR System (Applied Biosystems). Each sample was run in triplicate alongside with the reference gene. For validation of gene expression levels from peripheral blood samples from groups #1-3 (Figure 1B-C), *PPIB* (Hs00168719_m1) was used as reference gene [11,39]. In the qPCR experiments using blood RNA-derived samples from group #4 (Figure 1D)

Author Manuscript

and RNA from human brain samples (Figure 1E), *TRAP1* (Hs00972326_m1) [39] and *GAPDH* (Hs02758991_g1) were used as reference genes, respectively. Relative expression values were normalized to the respective reference gene using the $2^{-\Delta Ct}$ method[40] in DataAssist v3.0 software (Applied Biosystems).

Western Blotting

Protein extracts from DCN and cerebral cortex tissues were obtained by homogenization of brain tissue in cold PBS containing protease inhibitors (cOmplete, Roche) and phosphatase inhibitors (PhosSTOP, Roche), followed by sonication and centrifugation. The supernatant was collected (PBS-soluble fraction) and stored at -80°C. The pellet was resuspended in 1% sodium lauroyl sarcosinate (sarkosyl, Sark)/PBS, sonicated, centrifuged and the supernatant (sarkosyl-soluble fraction) was stored at -80°C. Total protein concentrations from PBS-soluble (soluble) and sarkosyl-soluble (insoluble) fractions were assessed as previously reported [41]. Total protein lysates (50µg) were resolved on 4-20% SDS-PAGE gels, and corresponding PVDF membranes were incubated overnight at 4°C with various antibodies: anti-DDIT4 (1:1000, ab191871, Abcam), anti-TRIM13 (1:1000, MBS7045610, MyBioSource), anti-P2RY13 (1:1000, MBS7002276, MyBioSource), anti-MJD antibody (1:10000 [42]), and anti-GAPDH (1:30000, MAB374, Millipore). Bound primary antibodies were visualized as previously described [41]. Film band intensity was quantified by densitometry using ImageJ.

Statistical analysis

Descriptive statistical analysis was used to explore the demographic, genetic and clinical data of MJD subjects. Normal distribution of continuous variables was tested using the Shapiro-Wilk test, and nonparametric tests were used when normality assumption was violated. Comparison between two categorical variables (sex/groups) was tested using a chi-square test, and the

Author Manuscript

comparison of the age at blood collection and of the expanded $(CAG)_n$ size between groups was tested using Kruskal-Wallis or Mann-Whitney U (two-tailed) tests.

Comparisons of expression levels between groups: *Blood samples* – comparison of transcripts levels between groups in Azorean cohorts (group #1 and group #2) was performed using a Generalized Linear Model with Bonferroni correction for multiple comparisons whenever applicable (covariate: age at blood collection) or using Mann-Whitney U (two-tailed) test in the Brazilian cohort (group #3). Comparison of transcript levels between PC or EP subjects and age- and sex-matched paired controls (Azorean cohort, group #4) was performed using Wilcoxon Signed-rank (two-tailed) test. A receiver operating characteristic (ROC) curve analysis was executed to determine the ability of each transcript abundance to differentiate: i) PC and EP subjects from age- and sex-matched paired controls; and ii) patients with more than five years of disease duration from controls. ROC curve analysis was also performed for the combination of the genes using the predicted probabilities obtained by multiple logistic regression. ROC curve accuracy was measured by the area under the curve (AUC), 95% confidence interval (CI), *p*-value; AUC values range [0 - 1.00] (0.50 is equivalent to randomly classifying subjects, and 1.00 is equivalent to perfectly classifying subjects). Youden Index [43] was used to determine the optimal cut-off value for each transcript abundance to differentiate the three groups of MJD subjects (PC, EP and patients > 5 years of disease duration) from their respective controls. *Post-mortem human brain samples* – comparison of transcript and protein levels between groups was performed using a Generalized Linear Model, adjusted for the age at death when this variable had an effect in the model.

Association with demographic, genetic and clinical MJD subjects' features: *Blood samples* – the relationship between transcript levels and MJD subjects' features was evaluated using Spearman´s rank correlation (two-tailed), and partial correlation (two-tailed) was used to adjust for covariates (expanded CAG repeat and/or disease duration) when necessary. The relationship

Author Manuscript

between transcript levels and MJD subjects features was tested in patients representing several stages of the disease, and in early patients.

Prism 8.0.0 was used to identify the extreme outliers (ROUT method Q=1%) that were then excluded from data analysis, to perform the ROC curve analysis, and to generate all the graphs. Other statistical procedures were performed using IBM SPSS Statistics 25 (IBM). Statistical significance was set at $p<0.05$.

Results

***DDIT4*, *TRIM13* and *P2RY13* transcript levels are altered in blood samples from two cohorts of MJD patients.**

To identify transcriptional changes in MJD, a gene expression microarray was performed using peripheral blood RNA samples from preclinical MJD subjects, MJD patients, and controls (group #1, Figure 1). About 9400 probes (of a total of 47231 probes) corresponding to 5209 genes passed the quality control. Comparison of blood transcript levels between the three biological groups (PC vs. controls, patients vs. controls, PC vs. patients) identified 77 genes with significant differences ($q<0.05$) in at least one comparison (data not shown, but available upon request to the authors). Among these dysregulated genes, 24 genes were selected to be technically validated by qPCR in the same blood RNA samples used in the microarray (group #1, Figure 1) (Supplementary Table 3). Of these 24 genes, *DDIT4* (DNA Damage Inducible Transcript 4), *TRIM13* (tripartite motif (TRIM) containing 13), *S100P* (S100 calcium binding protein P), *SVBP* (small vasohibin binding protein) and *UPS49* (ubiquitin specific peptidase 49) maintained their dysregulation pattern in independent samples from Azorean MJD patients (group #2, Figure 1), and were then further analyzed in samples from MJD patients and controls of an independent Brazilian cohort (group #3, Figure 1) (Figure 2; Supplementary Table 4). Levels of blood *P2RY13* (purinergic receptor P2Y13) transcripts, previously shown to be increased in Azorean MJD patients [11], were also investigated in samples from the Brazilian cohort (Figure 2). In summary,

Author Manuscript

Author Manuscript

the three genes (*DDIT4*, *TRIM13*, and *P2RY13*) displayed a consistent expression behavior in blood samples from MJD patients compared to controls in the two independent cohorts of different populational origins (Figure 2): the levels of *DDIT4* were decreased, whereas the levels of *TRIM13* and *P2RY13* were increased in MJD patients. Moreover, *TRIM13* transcript levels were increased in Azorean preclinical MJD subjects compared with respective controls (Figure 2). No sex differences were found for blood abundance of these three transcripts in all studied cohorts (data not shown).

Blood transcript levels of *DDIT4* and *TRIM13* correlate with the expanded $(CAG)_n$ size and *P2RY13* levels correlate with the age at onset of MJD patients.

Blood *DDIT4*, *TRIM13* and *P2RY13* transcript levels were correlated with demographic, genetic, and clinical features of MJD patients (groups #1-3, Figure 1; Supplementary Table 5). *DDIT4* and *TRIM13* levels inversely correlated with the CAG repeat number of the expanded *ATXN3* allele (rho=-0.290 and p=0.027, and rho=-0.330 and p=0.015, respectively), and *P2RY13* levels positively correlated with the age at disease onset (rho=0.366 and p=0.004) (Supplementary Table 5).

Blood levels of *DDIT4*, *TRIM13* and *P2RY13* transcripts are altered in the initial stages of MJD.

To determine the potential use of *DDIT4*, *TRIM13* and *P2RY13* transcript levels as blood-based biomarkers of initial stages of MJD, their abundance was evaluated in preclinical subjects, early-stage patients (< 5 years of disease duration) and age- and sex-matched paired controls (group #4, Figure 1D) (Figure 3). *DDIT4* levels were decreased whereas *TRIM13* and *P2RY13* levels were increased in both PC and EP subjects (Figure 3). These results observed for early-stage patients (Figure 1D) are in accordance with those obtained for the Azorean and Brazilian cohorts of MJD patients (groups #2 and 3, respectively, Figure 1C) that comprise subjects in a wider range of disease stages (up to 25 years after onset) (Figure 2). Furthermore, when correlating the

transcript levels of these three genes with the expanded $(CAG)_n$ size and the clinical features of early-stage patients, *TRIM13* levels inversely correlated with the age at disease onset (rho=-0.472, *p*=0.004) (Supplementary Table 6), suggesting that the differential blood *TRIM13* expression may be associated with disease severity.

Blood transcript levels of *DDIT4* and *TRIM13* differentiate MJD subjects from controls with high accuracy.

Next, the ability of blood transcript levels of *DDIT4*, *TRIM13* and *P2RY13* to differentiate MJD subjects from matched controls was investigated in various stages of the disease: i) in MJD subjects at initial stages of the disease (PC and EP subjects), and ii) in MJD patients with more than five years of disease duration (Figure 4). *TRIM13* and *DDIT4* levels differentiated with very high accuracy preclinical subjects from controls (*TRIM13*: AUC=0.99 (0.97-1.00), *p*<0.001; *DDIT4*: AUC=0.94 (0.86-1.00), *p*<0.001), and early-stage patients from controls (*TRIM13*: AUC=0.92 (0.85-0.98), *p*<0.001; *DDIT4*: AUC=0.93 (0.87-0.98), *p*<0.001) (Figure 4). On the other hand, *P2RY13* levels differentiated preclinical subjects and early-stage patients from controls with moderate (AUC=0.78 (0.63-0.93), *p*=0.004) and low accuracy (AUC=0.68 (0.55-0.81), *p*=0.010), respectively (Figure 4). Importantly, the combination of *DDIT4* and *TRIM13* levels perfectly differentiated preclinical subjects from controls (AUC=1.00 (1.00-1.00), *p*<0.001). In addition, the combination of *DDIT4* and *TRIM13* levels also improved the ability to differentiate early-stage patients from controls (AUC=0.96 (0.92-1.00), *p*<0.001) (Figure 4), while no improvement was seen when *P2RY13* abundance was added to the other two genes (AUC=0.96 (0.91-1.00), *p*<0.001). Noteworthy, blood *TRIM13* transcript levels further differentiated with good accuracy MJD patients with a disease duration longer than five years from controls (AUC=0.85 (0.77-0.93), *p*<0.001), and the combination of *DDIT4* and *TRIM13* levels improved the ability to differentiate these two groups (AUC=0.90 (0.83-0.96), *p*<0.001) (Figure 4). The optimal cut-off values for

Author Manuscript

DDIT4, *TRIM13*, and *P2RY13* transcript levels to differentiate MJD subjects in several disease stages from controls are summarized in Supplementary Table 7.

DDIT4, TRIM13 and P2RY13 protein levels are dysregulated in brain samples from MJD patients.

DDIT4, *TRIM13*, and *P2RY13* expression was assessed at the transcript and protein levels in a set of postmortem brain samples from MJD patients and controls. The abundance of *DDIT4*, *TRIM13*, and *P2RY13* transcripts in the three analyzed brain regions (the severely affected pons and DCN and the cerebral cortex, which shows a lower degree of degeneration in MJD) was similar in MJD patients and controls (Figure 5).

At the protein level, the abundance of soluble TRIM13 was significantly increased in the cerebral cortex of MJD patients compared to controls(p=0.0043) (Figure 6; Supplementary Figure 1).No further differences were detected for insoluble or soluble TRIM13, DDIT4, and P2RY13 protein levels in the DCN or cerebral cortex of MJD patients relative to controls (Figure 6).

Discussion

This study aimed to identify transcriptional changes in peripheral blood of MJD subjects that could be used as prospective biomarkers of MJD, especially of its initial stages. Our unbiased genome-wide expression microarray confirmed the previously reported existence of global transcription dysregulation in blood from MJD subjects[11,14]. To investigate whether the transcriptional alterations found at the periphery are conserved in the tissue primarily affected in MJD, the transcript and protein levels of three differentially expressed genes in the blood were assessed in post-mortem brain samples from MJD patients and controls.

Three genes – *DDIT4*, *TRIM13* and *P2RY13* – were identified here as being consistently dysregulated in blood of MJD carriers in various stages of disease, including in preclinical and early disease stages. In a previous RNA-seq study conducted by our group[14], using fewer MJD

Author Manuscript

Author Manuscript

preclinical subjects with mixed genetic backgrounds no significant differences in transcriptional levels of *DDIT4*, *TRIM13,* and *P2RY13* were observed compared to controls. Noteworthy, in that study[14], candidate transcriptional biomarkers were identified in patients, not by comparing expression levels between patients and controls, but by correlating gene expression levels with the score of the Scale for the assessment and rating of ataxia (SARA). Interestingly blood *DDIT4* transcript levels were negatively correlated with patients' SARA score (Log Fold change=-0.40; *p*=0.0164). Our results showed that individual blood transcript levels of *DDIT4* and *TRIM13* can differentiate with very high accuracy (AUC>0.90) both PC and EP subjects from age- and sex-matched paired controls. Remarkably, the combination of *DDIT4* and *TRIM13* blood transcript levels fully differentiated preclinical subjects from controls (AUC=1.00), and further differentiated with high accuracy patients with more than five years of disease duration from controls (AUC=0.90). To date, NfL [44–48] and mutant ATXN3 protein levels have been considered the blood-based molecular biomarkers that better differentiate MJD subjects from control subjects and correlate with clinical and genetic features of the disease [47,49,50]. However, the use of blood NfL and mutant ATXN3 levels as biomarkers of MJD shows some limitations: i) blood NfL levels are a general biomarker of neurodegeneration (not MJD-specific) that is also affected by other factors such as age, body-mass index, diabetes and cardiovascular risk factors [51]; and ii) blood mutant ATXN3 levels may not be useful to assess the efficacy of disease-modifying therapeutic agents other than agents targeting *ATXN3* gene products. In addition, only some of the aforementioned NfL studies assessed whether blood-based molecular alterations differentiate premanifest subjects [44–46,48,50] and early-stage patients [44] from controls. Noteworthy, prior to disease onset, individually or combined blood *DDIT4* and *TRIM13* transcript levels, differentiated MJD carriers from healthy controls with higher accuracy (*DDIT4*: 94%, *TRIM13*: 99% and *DDIT4+TRIM13*: 100%, respectively) than serum and plasma NfL levels (79-89%) [44,45,48]. Concerning the early disease stage, the combination of *DDIT4* and *TRIM13* transcript levels and serum NfL levels alone [44] differentiate early-stage patients from controls with similar

Author Manuscript

accuracy (96% and 98%, respectively). These findings highlight the potential of *DDIT4* and *TRIM13* transcript levels as blood-based biomarkers of MJD, particularly of preclinical and early symptomatic stages of disease. Interestingly, no changes of blood *DDIT4*, *TRIM13* and *P2RY13* transcripts have been found in large cohorts of patients with Huntington's disease [52–56] or Parkinson's disease [57] or Friedreich's ataxia [58] compared with their correspondent healthy controls, suggesting that alteration of blood abundance of these transcripts might be specific of MJD. Supporting the hypothesis that these expression differences are meaningful and linked to the disease process, the expression levels of *DDIT4*, *TRIM13* and *P2RY13* genes correlated with patients' age at onset and/or the expanded $(CAG)_n$ number. Moreover, while the number of assessed post-mortem brain samples in this study was low, we observed increased levels of soluble TRIM13 protein in the cerebral cortex from MJD patients compared to controls (no changes were found at the mRNA level), further suggesting a potential involvement of this protein in MJD-associated mechanisms.

DDIT4 protein localizes mainly in the cytoplasm, and is primarily expressed in T-cells, macrophages, and astrocytes [59]. DDIT4 protein plays a role in regulating cell growth, proliferation, and survival by inhibiting the activity of mTORC1 (mammalian target of rapamycin complex 1) [60–62]. DDIT4 levels are induced by cellular stress, and prolonged DDIT4 expression is associated with apoptosis, excessive production of reactive oxygen species, and inflammation activation [63]. Because reduced DDIT4 abundance results in loss of mitochondrial integrity, leading to increased inflammatory response [64,65], our observed reduction of blood *DDIT4* transcript levels in MJD carriers seems to be in line with the impaired mitochondrial function previously shown in blood of MJD [17,18]. In contrast, the levels of DDIT4 and P2RY13 proteins in the highly affected brain region (DCN) and the less affected region (cerebral cortex) in MJD were similar in patients and controls. Future studies including a higher number of brain samples per biological group would be important to validate these results.

Author Manuscript

TRIM13 is expressed in immune cells and in neuronal and glial cells [59]. TRIM13 protein is a positive regulator of nuclear factor-kappa B (NF-kB) signaling, inducing the production of cytokines and inflammatory mediators [66]. While *TRIM13* has not been associated with any neurodegenerative disease, the TRIM13 protein has been reported to be involved in processes that are altered in MJD including the elimination of endoplasmic reticulum (ER)-associated protein degradation substrates [67], regulation of autophagy [68,69], and cell death during ER stress [70]. In fact, TRIM family members are crucial players in eliminating misfolded proteins [71], and some TRIM proteins have been associated with neurodegenerative diseases, namely TRIM19 in SCA1 [35] and TRIM11 in Parkinson's and Alzheimer diseases [36,37]. Comparing with controls, abundance of blood *TRIM13* transcripts was increased in MJD subjects suggesting peripheral inflammation aligning with the reported increase of inflammatory markers in MJD blood [16]. *TRIM13* transcript levels inversely correlated with the expanded CAG repeat in the combined group of Azorean and Brazilian MJD patients, and with the age of onset of early-stage Azorean patients. In post-mortem brains, soluble TRIM13 protein levels were increased in MJD patients' cerebral cortex compared to controls. Overall, we speculate that the increase of *TRIM13* expression levels in blood and post-mortem cerebral cortex samples from MJD subjects could possibly reflect the attempt of cells to promote the clearance of misfolded proteins and aggregates during ER stress and/or to induce apoptosis if the ER stress conditions persist.

P2RY13 encodes the P2Y purinoceptor 13 protein that belongs to the large G-protein coupled receptors (GPCR) family of membrane proteins [72]. *P2RY3* is mainly expressed in macrophages cells and in microglial cells [59]. P2RY13 membrane protein induces the expression and the release of several proinflammatory cytokines [73]. In this work, blood *P2RY13* transcript levels were increased in Brazilian MJD patients, and in Azorean preclinical MJD subjects and early-stage patients, which is in agreement with previously reported observations in a group of Azorean MJD patients in several stages of the disease up to 30 years after onset [11]. Interestingly, blood *P2RY13* transcript abundance is directly correlated with the age at onset of MJD patients

Author Manuscript

suggesting a link with MJD pathogenesis. Overall, the increase of *P2RY13* abundance in the blood of MJD subjects may also indicate an inflammatory response in this tissue [74].

Here, the relevance of altered abundance of *DDIT4*, *TRIM13*, and *P2RY13* gene products in MJD subjects is determined by: i) consistent transcriptional behaviors in blood of MJD subjects from two cohorts of different genetic backgrounds, which are markedly distinct from the ones observed in healthy controls; ii) the correlation between transcript levels and patients' clinical and/or genetic features; and iii) altered soluble TRIM13 protein levels in the cerebral cortex of MJD patient. Importantly, this study reveals that the abundance of blood *DDIT4* and *TRIM1*3 transcripts differentiates MJD subjects (from preclinical to symptomatic stages) from controls, especially in the preclinical and early symptomatic stages. No differences were found between the blood abundances of *DDIT4* and *TRIM13* transcripts in Azorean PC and P subjects (Figure2), suggesting that these transcript levels could be diagnostic biomarkers of MJD. However, further studies are needed to confirm the potential of these molecules as MJD transcriptional therapeutic or diagnostic biomarkers, such as testing their levels throughout disease progression (longitudinal studies) and assessing how transcript levels correlate with other clinical features of the disease.

Although MJD is known as a neurological disease, the *ATXN3* gene is broadly expressed in several cell types from peripheral tissues and the central nervous system, as reported in various studies that have shown changes in the blood of MJD carriers [11–15]. The blood transcriptional changes observed in MJD subjects reflect disease mechanisms that, regardless of being or not preserved in the brain, are hallmarks of the disease and can be used as MJD biomarkers. In addition, the alterations in the transcript levels of *DDIT4*, *TRIM13* and *P2RY13* in blood samples, together with the changes of TRIM13 protein levels in brain samples of MJD patients suggest that these genes/proteins could play a role in the disease pathogenesis in the blood and/or the brain. The involvement of these genes in processes known to be dysregulated in MJD, such as inflammation/neuroinflammation, further supports this hypothesis. In the future, it would be

Author Manuscript

important to evaluate the abundance of DDIT4, TRIM13 and P2RY13 protein levels in blood samples from MJD subjects as these readouts might provide additional usefulness as biomarkers of this disease.

Overall, future assessment of blood *DDIT4* and *TRIM13* transcript levels in subjects from MJD cohorts of different geographical regions and genetic backgrounds and subjects affected with other neurodegenerative disorders would inform us about their solid potential as specific biomarkers of MJD or as general biomarkers of neurodegenerative disorders.

Acknowledgements

We are grateful to the MJD subjects and their relatives as well as to the healthy volunteers for participating in this study. The Michigan brain bank at the University of Michigan is partially supported by the NIH/NIA funded Michigan Alzheimer's Disease Center (P30AG053760 and P30AG072931). This work was funded by FEDER funds through the Operational Competitiveness Programme (COMPETE), by National Funds through Fundação para a Ciência e Tecnologia (FCT) under the project FCOMP-01-0124-FEDER-028753 (PTDC/DTP/PIC/0370/2012), and by discretionary funds of the University of Michigan to MCC. This work was also funded by ESMI, an EU Joint Programme - Neurodegenerative Disease Research (JPND) project (see www.jpnd.eu); ESMI project is supported through the following funding organizations under the aegis of JPND: Germany, Federal Ministry of Education and Research (BMBF; funding codes 01ED1602A/B); Netherlands, The Netherlands Organization for Health Research and Development; Portugal, FCT (JPCOFUND/0002/2015); United Kingdom, Medical Research Council. AFF was supported by a PhD fellowship (SFRH/BD/121101/2016) funded by FCT, República Portuguesa/Ciência, Tecnologia e Ensino Superior, Portugal 2020, União Europeia/Fundo Social Europeu and POR_NORTE. MR is supported by FCT (CEECIND/03018/2018/CP1556/CT0009). CB is supported by Alzheimer's Research UK (ARUK-

RF2019B-005) and the Multiple System Atrophy Trust (UK). MCC is supported by the Heeringa Ataxia Research Fund (University of Michigan).

Authors' contributions

AFF, MR, CB, MCC, and ML contributed to the study's conception and design; AFF, MR, EDS, LL, JV, TK, MLSP, LBJ, MCC, and ML contributed to data acquisition and analysis; and AFF, MCC, and ML contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors declare that they have no competing interests.

Data availability

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

References

1. Klockgether T, Mariotti C, Paulson HL. Spinocerebellar ataxia [Internet]. Nat. Rev. Dis. Prim. 2019;5(1):24.Available from: http://dx.doi.org/10.1038/s41572-019-0074-3
2. Riess O, Rüb U, Pastore A, et al. SCA3: neurological features, pathogenesis and animal models. [Internet]. Cerebellum 2008;7(2):125–37.Available from: http://www.ncbi.nlm.nih.gov/pubmed/18418689
3. Seidel K, Siswanto S, Brunt ERP, et al. Brain pathology of spinocerebellar ataxias. Acta Neuropathol. 2012;124(1):1–21.
4. Maciel P, Costa MC, Ferro A, et al. Improvement in the molecular diagnosis of Machado-Joseph disease. [Internet]. Arch. Neurol. 2001;58(11):1821–7.Available from: http://www.ncbi.nlm.nih.gov/pubmed/11708990

Author Manuscript

Author Manuscript

5. Wu X, Liao X, Zhan Y, et al. Microstructural Alterations in Asymptomatic and Symptomatic Patients with Spinocerebellar Ataxia Type 3: A Tract-Based Spatial Statistics Study. Front. Neurol. 2017;8:714.

6. Rezende TJR, de Paiva JLR, Martinez ARM, et al. Structural signature of SCA3: From presymptomatic to late disease stages. Ann. Neurol. 2018;84(3):401–408.

7. Globas C, du Montcel ST, Baliko L, et al. Early symptoms in spinocerebellar ataxia type 1, 2, 3, and 6. Mov. Disord. 2008;23(15):2232–2238.

8. Raposo M, Vasconcelos J, Bettencourt C, et al. Nystagmus as an early ocular alteration in Machado-Joseph disease (MJD/SCA3). BMC Neurol. 2014;14:17.

9. Costa M do C, Paulson HL. Toward understanding Machado–Joseph disease [Internet]. Prog. Neurobiol. 2012;97(2):239–257.Available from: http://linkinghub.elsevier.com/retrieve/pii/S0301008211002127

10. Evers MM, Toonen LJA, Van Roon-Mom WMC. Ataxin-3 protein and RNA toxicity in spinocerebellar ataxia type 3: Current insights and emerging therapeutic strategies. Mol. Neurobiol. 2014;49(3):1513–1531.

11. Raposo M, Bettencourt C, Maciel P, et al. Novel candidate blood-based transcriptional biomarkers of Machado-Joseph disease [Internet]. Mov. Disord. 2015;30(7):968–975.Available from: http://doi.wiley.com/10.1002/mds.26238

12. Kazachkova N, Raposo M, Ramos A, et al. Promoter Variant Alters Expression of the Autophagic BECN1 Gene: Implications for Clinical Manifestations of Machado-Joseph Disease. Cerebellum 2017;16(5–6):957–963.

13. Raposo M, Bettencourt C, Ramos A, et al. Promoter Variation and Expression Levels of Inflammatory Genes IL1A, IL1B, IL6 and TNF in Blood of Spinocerebellar Ataxia Type 3 (SCA3) Patients. NeuroMolecular Med. 2017;19(1):41–45.

14. Raposo M, Hübener-Schmid J, Ferreira AF, et al. Blood transcriptome sequencing identifies biomarkers able to track disease stages in spinocerebellar ataxia type 3. Brain

2023;

15. Ferreira AF, Raposo M, Shaw ED, et al. Tissue-Specific Vulnerability to Apoptosis in Machado-Joseph Disease. Cells 2023;12(10)

16. da Silva Carvalho G, Saute JAM, Haas CB, et al. Cytokines in Machado Joseph Disease/Spinocerebellar Ataxia 3 [Internet]. Cerebellum 2016;15(4):518–525.Available from: http://dx.doi.org/10.1007/s12311-015-0719-z

17. de Assis AM, Saute JAM, Longoni A, et al. Peripheral oxidative stress biomarkers in spinocerebellar ataxia type 3/Machado-Joseph disease. Front. Neurol. 2017;8(SEP):1–8.

18. Raposo M, Ramos A, Santos C, et al. Accumulation of Mitochondrial DNA Common Deletion Since The Preataxic Stage of Machado-Joseph Disease. Mol. Neurobiol. 2019;56(1):119–124.

19. Rubinsztein DC, Orr HT. Diminishing return for mechanistic therapeutics with neurodegenerative disease duration?: There may be a point in the course of a neurodegenerative condition where therapeutics targeting disease-causing mechanisms are futile. Bioessays 2016;38(10):977–980.

20. Maas RPPWM, Teerenstra S, Lima M, et al. Differential Temporal Dynamics of Axial and Appendicular Ataxia in SCA3. Mov. Disord. 2022;37(9):1850–1860.

21. Klockgether T, Lüdtke R, Kramer B, Abele M, Bürk K, Schöls L, Riess O, Laccone F, Boesch S, Lopes-Cendes I, Brice A, Inzelberg R, Zilber N DJ. The natural history of degenerative ataxia: a retrospective study in 466 patients [Internet]. Brain 1998;121(4):589–600.Available from: https://academic.oup.com/brain/article-lookup/doi/10.1093/brain/121.4.589

22. Kieling C, Rieder CRM, Silva ACF, et al. A neurological examination score for the assessment of spinocerebellar ataxia 3 (SCA3) [Internet]. Eur. J. Neurol. 2008;15(4):371–376.Available from: http://doi.wiley.com/10.1111/j.1468-1331.2008.02078.x

Author Manuscript

Author Manuscript

23. Raposo M, Ramos A, Bettencourt C, Lima M. Replicating studies of genetic modifiers in spinocerebellar ataxia type 3: Can homogeneous cohorts aid? Brain 2015;138(12):e398.

24. Bettencourt C, Fialho RN, Santos C, et al. Segregation distortion of wild-type alleles at the Machado-Joseph disease locus: a study in normal families from the Azores islands (Portugal). [Internet]. J. Hum. Genet. 2008;53(4):333–9.[cited 2012 Mar 8] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18286225

25. Rüb U, Brunt ER, Deller T. New insights into the pathoanatomy of spinocerebellar ataxia type 3 (Machado-Joseph disease). Curr. Opin. Neurol. 2008;21(2):111–116.

26. de Rezende TJR, D'Abreu A, Guimaraes RP, et al. Cerebral cortex involvement in Machado-Joseph disease. Eur. J. Neurol. 2015;22(2):277–83, e23-4.

27. Arruda WO, Meira AT, Ono SE, et al. Volumetric MRI Changes in Spinocerebellar Ataxia (SCA3 and SCA10) Patients. Cerebellum 2020;19(4):536–543.

28. Ashraf NS, Duarte-Silva S, Shaw ED, et al. Citalopram Reduces Aggregation of ATXN3 in a YAC Transgenic Mouse Model of Machado-Joseph Disease. Mol. Neurobiol. 2019;56(5):3690–3701.

29. Petryszak R, Keays M, Tang YA, et al. Expression Atlas update—an integrated database of gene and protein expression in humans, animals and plants [Internet]. Nucleic Acids Res. 2015;44(D1):D746–D752.Available from: https://doi.org/10.1093/nar/gkv1045

30. MacDonald JR, Ziman R, Yuen RKC, et al. The Database of Genomic Variants: a curated collection of structural variation in the human genome. Nucleic Acids Res. 2014;42(Database issue):D986-92.

31. Hong S, Kim S-J, Ka S, et al. USP7, a ubiquitin-specific protease, interacts with ataxin-1, the SCA1 gene product. Mol. Cell. Neurosci. 2002;20(2):298–306.

32. Silvestroni A, Faull RLM, Strand AD, Möller T. Distinct neuroinflammatory profile in post-mortem human Huntington's disease. Neuroreport 2009;20(12):1098–1103.

33. He W-T, Zheng X-M, Zhang Y-H, et al. Cytoplasmic Ubiquitin-Specific Protease 19

(USP19) Modulates Aggregation of Polyglutamine-Expanded Ataxin-3 and Huntingtin through the HSP90 Chaperone. PLoS One 2016;11(1):e0147515.

34. Evert BO, Vogt IR, Kindermann C, et al. Inflammatory genes are upregulated in expanded ataxin-3-expressing cell lines and spinocerebellar ataxia type 3 brains. J. Neurosci. 2001;21(15):5389–5396.

35. Guo L, Giasson BI, Glavis-Bloom A, et al. A cellular system that degrades misfolded proteins and protects against neurodegeneration. Mol. Cell 2014;55(1):15–30.

36. Zhu G, Harischandra DS, Ghaisas S, et al. TRIM11 Prevents and Reverses Protein Aggregation and Rescues a Mouse Model of Parkinson's Disease. Cell Rep. 2020;33(9):108418.

37. Zhang Z-Y, Harischandra DS, Wang R, et al. TRIM11 protects against tauopathies and is down-regulated in Alzheimer's disease [Internet]. Science (80-.). 2023;381(6656):eadd6696.Available from: https://doi.org/10.1126/science.add6696

38. Dietzsch J, Gehlenborg N, Nieselt K. Mayday-a microarray data analysis workbench [Internet]. Bioinformatics 2006;22(8):1010–1012.Available from: https://doi.org/10.1093/bioinformatics/btl070

39. Ferreira AF, Raposo M, Vasconcelos J, et al. Selection of Reference Genes for Normalization of Gene Expression Data in Blood of Machado-Joseph Disease/Spinocerebellar Ataxia Type 3 (MJD/SCA3) Subjects. J. Mol. Neurosci. 2019;3(69):450–455.

40. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method [Internet]. Methods 2001;25(4):402–408.Available from: http://linkinghub.elsevier.com/retrieve/pii/S1046202301912629

41. Costa M do C, Ashraf NS, Fischer S, et al. Unbiased screen identifies aripiprazole as a modulator of abundance of the polyglutamine disease protein, ataxin-3 [Internet]. Brain 2016;139(11):2891–2908.Available from:

Author Manuscript

https://pubmed.ncbi.nlm.nih.gov/27645800

42. Paulson HL, Das SS, Crino PB, et al. Machado-Joseph disease gene product is a cytoplasmic protein widely expressed in brain. Ann. Neurol. 1997;41(4):453–462.

43. Youden WJ. Index for rating diagnostic tests [Internet]. Cancer 1950;3(1):32–35.Available from: https://onlinelibrary.wiley.com/doi/10.1002/1097-0142(1950)3:1%3C32::AID-CNCR2820030106%3E3.0.CO;2-3

44. Li Q, Dong Y, Yang L, et al. Neurofilament light chain is a promising serum biomarker in spinocerebellar ataxia type 3. Mol. Neurodegener. 2019;19(39)

45. Peng Y, Zhang Y, Chen Z, et al. Association of serum neurofilament light and disease severity in patients with spinocerebellar ataxia type 3 [Internet]. Neurology 2020;95(22):e2977–e2987.Available from: http://www.neurology.org/lookup/doi/10.1212/WNL.0000000000010671

46. Wilke C, Haas E, Reetz K, et al. Neurofilaments in spinocerebellar ataxia type 3: blood biomarkers at the preataxic and ataxic stage in humans and mice [Internet]. EMBO Mol. Med. 2020;12(7):e11803.Available from: https://www.embopress.org/doi/10.15252/emmm.201911803

47. Prudencio M, Garcia-Moreno H, Jansen-West KR, et al. Toward allele-specific targeting therapy and pharmacodynamic marker for spinocerebellar ataxia type 3. Sci. Transl. Med. 2020;12(566)

48. Garcia-Moreno H, Prudencio M, Thomas-Black G, et al. Tau and neurofilament light-chain as fluid biomarkers in spinocerebellar ataxia type 3 [Internet]. Eur. J. Neurol. 2022;29(8):2439–2452.Available from: https://doi.org/10.1111/ene.15373

49. Gonsior K, Kaucher GA, Pelz P, et al. PolyQ-expanded ataxin-3 protein levels in peripheral blood mononuclear cells correlate with clinical parameters in SCA3: a pilot study [Internet]. J. Neurol. 2021;268(4):1304–1315.Available from: https://link.springer.com/10.1007/s00415-020-10274-y

Author Manuscript

50. Hübener-Schmid J, Kuhlbrodt K, Peladan J, et al. Polyglutamine-Expanded Ataxin-3: A Target Engagement Marker for Spinocerebellar Ataxia Type 3 in Peripheral Blood [Internet]. Mov. Disord. 2021;36(11):2675–2681.Available from: https://doi.org/10.1002/mds.28749

51. Barro C, Chitnis T, Weiner HL. Blood neurofilament light: a critical review of its application to neurologic disease [Internet]. Ann. Clin. Transl. Neurol. 2020;7(12):2508–2523.Available from: https://pubmed.ncbi.nlm.nih.gov/33146954

52. Borovecki F, Lovrecic L, Zhou J, et al. Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. 2005;102(31):11023–11028.

53. Runne H, Kuhn A, Wild EJ, et al. Analysis of potential transcriptomic biomarkers for Huntington's disease in peripheral blood. Proc. Natl. Acad. Sci. U. S. A. 2007;104(36):14424–14429.

54. Mastrokolias A, Ariyurek Y, Goeman JJ, et al. Huntington's disease biomarker progression profile identified by transcriptome sequencing in peripheral blood. Eur. J. Hum. Genet. 2015;23(10):1349–1356.

55. Hensman Moss DJ, Flower MD, Lo KK, et al. Huntington's disease blood and brain show a common gene expression pattern and share an immune signature with Alzheimer's disease. Sci. Rep. 2017;7:44849.

56. Andrade-Navarro MA, Mühlenberg K, Spruth EJ, et al. RNA Sequencing of Human Peripheral Blood Cells Indicates Upregulation of Immune-Related Genes in Huntington's Disease [Internet]. Front. Neurol. 2020;11Available from: https://www.frontiersin.org/journals/neurology/articles/10.3389/fneur.2020.573560

57. Craig DW, Hutchins E, Violich I, et al. RNA sequencing of whole blood reveals early alterations in immune cells and gene expression in Parkinson's disease. Nat. aging 2021;1(8):734–747.

Author Manuscript

58. Nachun D, Gao F, Isaacs C, et al. Peripheral blood gene expression reveals an inflammatory transcriptomic signature in Friedreich's ataxia patients [Internet]. Hum. Mol. Genet. 2018;27(17):2965–2977.Available from: https://doi.org/10.1093/hmg/ddy198

59. Karlsson M, Zhang C, Méar L, et al. A single-cell type transcriptomics map of human tissues. Sci. Adv. 2021;7(31)

60. Corradetti MN, Inoki K, Guan K-L. The stress-inducted proteins RTP801 and RTP801L are negative regulators of the mammalian target of rapamycin pathway. J. Biol. Chem. 2005;280(11):9769–9772.

61. Sofer A, Lei K, Johannessen CM, Ellisen LW. Regulation of mTOR and cell growth in response to energy stress by REDD1. Mol. Cell. Biol. 2005;25(14):5834–5845.

62. Malagelada C, Ryu EJ, Biswas SC, et al. RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. J. Neurosci. 2006;26(39):9996–10005.

63. Britto FA, Dumas K, Giorgetti-Peraldi S, et al. Is REDD1 a metabolic double agent? Lessons from physiology and pathology. Am. J. Physiol. Cell Physiol. 2020;319(5):C807–C824.

64. Ip WKE, Hoshi N, Shouval DS, et al. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages [Internet]. Science (80-.). 2017;356(6337):513–519.Available from: https://doi.org/10.1126/science.aal3535

65. Pan X, Zhang Z, Liu C, et al. Circulating levels of DDIT4 and mTOR, and contributions of BMI, inflammation and insulin sensitivity in hyperlipidemia. Exp. Ther. Med. 2022;24(5):666.

66. Huang B, Baek S-H. Trim13 Potentiates Toll-Like Receptor 2-Mediated Nuclear Factor kappaB Activation via K29-Linked Polyubiquitination of Tumor Necrosis Factor

Author Manuscript

Receptor-Associated Factor 6. Mol. Pharmacol. 2017;91(4):307–316.

67. Mikael Lerner, Martin Corcoran, Diana Cepeda, Michael L. Nielsen, Roman Zubarev, Fredrik Pontén, Mathias Uhlén, Sophia Hober, Dan Grandér OS. The RBCC Gene RFP2 (Leu5) Encodes a Novel Transmembrane E3 Ubiquitin Ligase Involved in ERAD. Mol. Biol. Cell 2007;18:1670–1682.

68. Tomar D, Singh R, Singh AK, et al. TRIM13 regulates ER stress induced autophagy and clonogenic ability of the cells [Internet]. Biochim. Biophys. Acta - Mol. Cell Res. 2012;1823(2):316–326.Available from: http://dx.doi.org/10.1016/j.bbamcr.2011.11.015

69. Ji CH, Kim HY, Heo AJ, et al. The N-Degron Pathway Mediates ER-phagy. Mol. Cell 2019;75(5):1058-1072.e9.

70. Tomar D, Prajapati P, Sripada L, et al. TRIM13 regulates caspase-8 ubiquitination, translocation to autophagosomes and activation during ER stress induced cell death. Biochim. Biophys. Acta 2013;1833(12):3134–3144.

71. Zhang L, Afolabi LO, Wan X, et al. Emerging Roles of Tripartite Motif-Containing Family Proteins (TRIMs) in Eliminating Misfolded Proteins [Internet]. Front. Cell Dev. Biol. 2020;8:802.Available from: https://www.frontiersin.org/article/10.3389/fcell.2020.00802

72. Communi D, Gonzalez NS, Detheux M, et al. Identification of a novel human ADP receptor coupled to G(i). J. Biol. Chem. 2001;276(44):41479–41485.

73. Liu P-W, Yue M-X, Zhou R, et al. P2Y(12) and P2Y(13) receptors involved in ADPβs induced the release of IL-1β, IL-6 and TNF-α from cultured dorsal horn microglia. J. Pain Res. 2017;10:1755–1767.

74. Duarte Lobo D, Nobre RJ, Oliveira Miranda C, et al. The blood-brain barrier is disrupted in Machado-Joseph disease/spinocerebellar ataxia type 3: evidence from transgenic mice and human post-mortem samples [Internet]. Acta Neuropathol. Commun. 2020;8(1):152.Available from: https://doi.org/10.1186/s40478-020-00955-0

Author Manuscript

Author Manuscript

Figure legends

Figure 1. Overview of the experimental workflow used in this study. Peripheral blood samples from MJD subjects and controls from the Azorean cohort were used in (A) a genome-wide expression microarray analysis and (B) technical validation by qPCR. (C) The expression levels were evaluated in two independent cohorts: (C.1) The expression levels of a set of dysregulated genes were assessed in a set of Azorean samples; and (C.2) genes considered as the most promising disease markers were evaluated in a set of patients and controls of the Brazilian cohort. (D) The expression levels of the validated genes were assessed in a set of Azorean PC subjects and early patients. (E) The expression of the validated genes was evaluated at the transcript and protein levels in post-mortem brain samples from MJD patients and controls. Human brain figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. PC=preclinical subjects; EP=early patients; P=patients; C=controls; CPC=controls of PC subjects; CEP=controls of EP.

Figure 2. Blood transcript levels of *DDIT4*, *TRIM13,* and *P2RY13* are consistently dysregulated in two independent MJD cohorts. Gene expression levels of *DDIT4*, *TRIM13, P2RY13 S100P*, *SVBP* and *USP49* in peripheral blood samples from MJD carriers (PC, preclinical subject, and P, patient)s and controls (C) from the Azorean cohort (group #2) and MJD patients (P) and controls (C) from the Brazilian cohort (group #3). Dots represent transcript levels for each individual. Graphs show the mean (Azores) or median (Brazil) and the 95% confidence interval of the $2^{-\Delta Ct}$ values.

Figure 3. Blood transcript levels of *DDIT4*, *TRIM13* and *P2RY13* are dysregulated in initial stages of the disease. Genes expression levels in peripheral blood samples from preclinical (PC) subjects

and early patients (EP), and age- and sex-matched paired controls (CPC, controls of preclinical subjects); CEP, controls of EP) from group #4. Dots represent transcript levels for each individual. Graphs show the median and the 95% confidence interval of the $2^{-\Delta Ct}$ values.

Figure 4. Blood transcript levels of *DDIT4* and *TRIM13* differentiate with very high accuracy MJD subjects from controls. Receiver operating characteristics curves show the ability of blood transcript levels of *DDIT4*, *TRIM13*, *P2RY13* and the combination of *DDIT4* and *TRIM13* levels to differentiate MJD subjects in different stages of the disease (PC, light gray line; EP, dark gray line; patients with > 5 years of disease duration, black line) from matched controls.

Figure 5. *DDIT4*, *TRIM13* and *P2RY13* transcript levels are similar in post-mortem brain of MJD patients and controls. Transcript levels in pons, dentate cerebellar nucleus (DCN) and cerebral cortex (cortex) from MJD patients (P) and controls (C). Dots represent transcript levels for each individual. Graphs show the mean and the standard error mean of the $2^{-\Delta Ct}$ values.

Figure 6. TRIM13 protein levels are altered in post-mortem brain of MJD patients. PBS-soluble (soluble) and sarkosyl-soluble (insoluble) protein fractions were analyzed in dentate cerebellar nucleus (DCN) and cerebral cortex (cortex) from MJD patients (P) and controls (C). Left panels show immunoblots of indicated proteins in soluble and insoluble protein fractions. Right panels display quantification of band intensity, with values normalized to GAPDH. Dots represent protein levels for each individual. Graphs show the mean and the standard error mean of protein relative to controls.

Author Manuscript

Author Manuscript

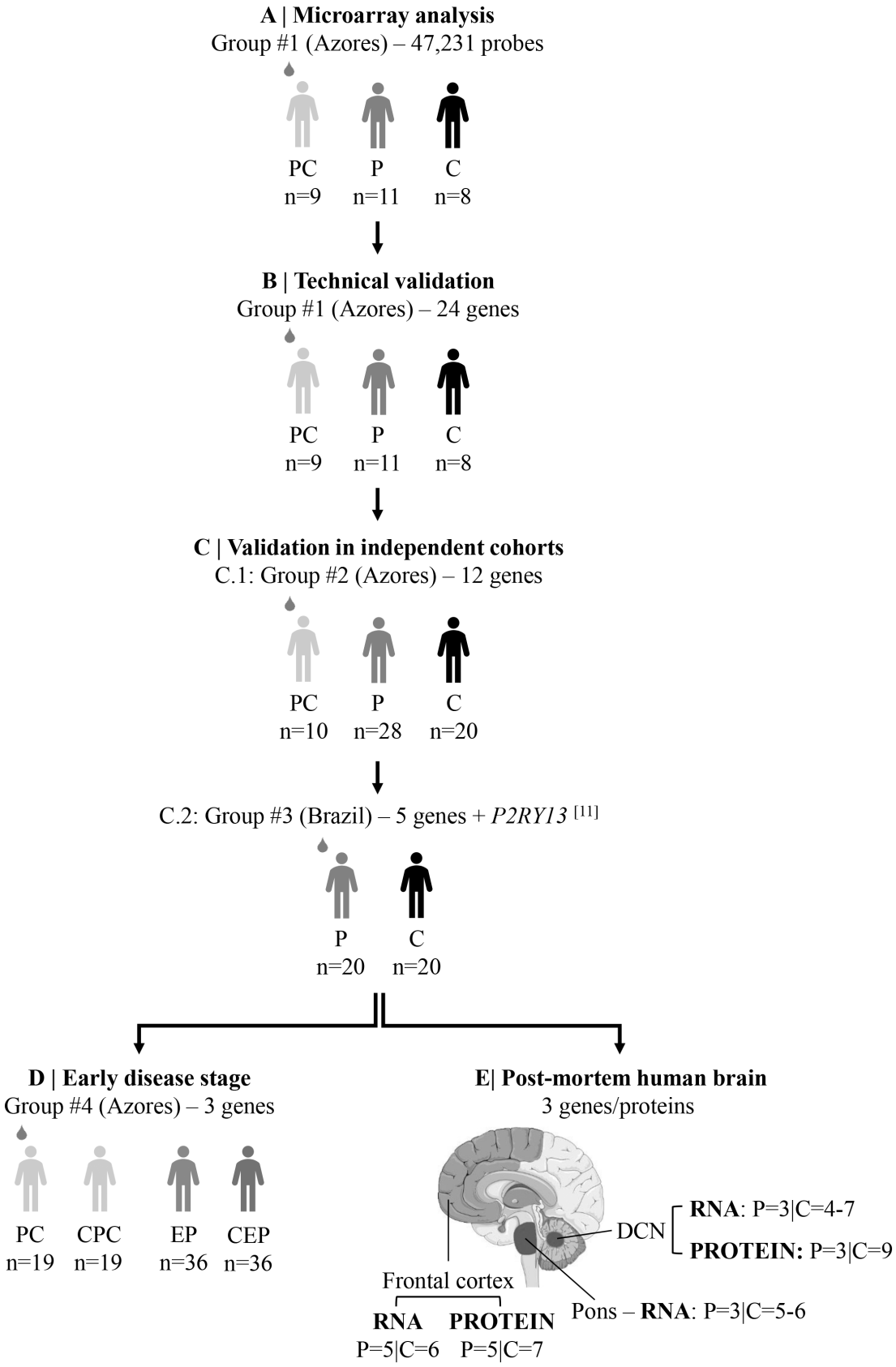


Figure 1.tif

Author Manuscript

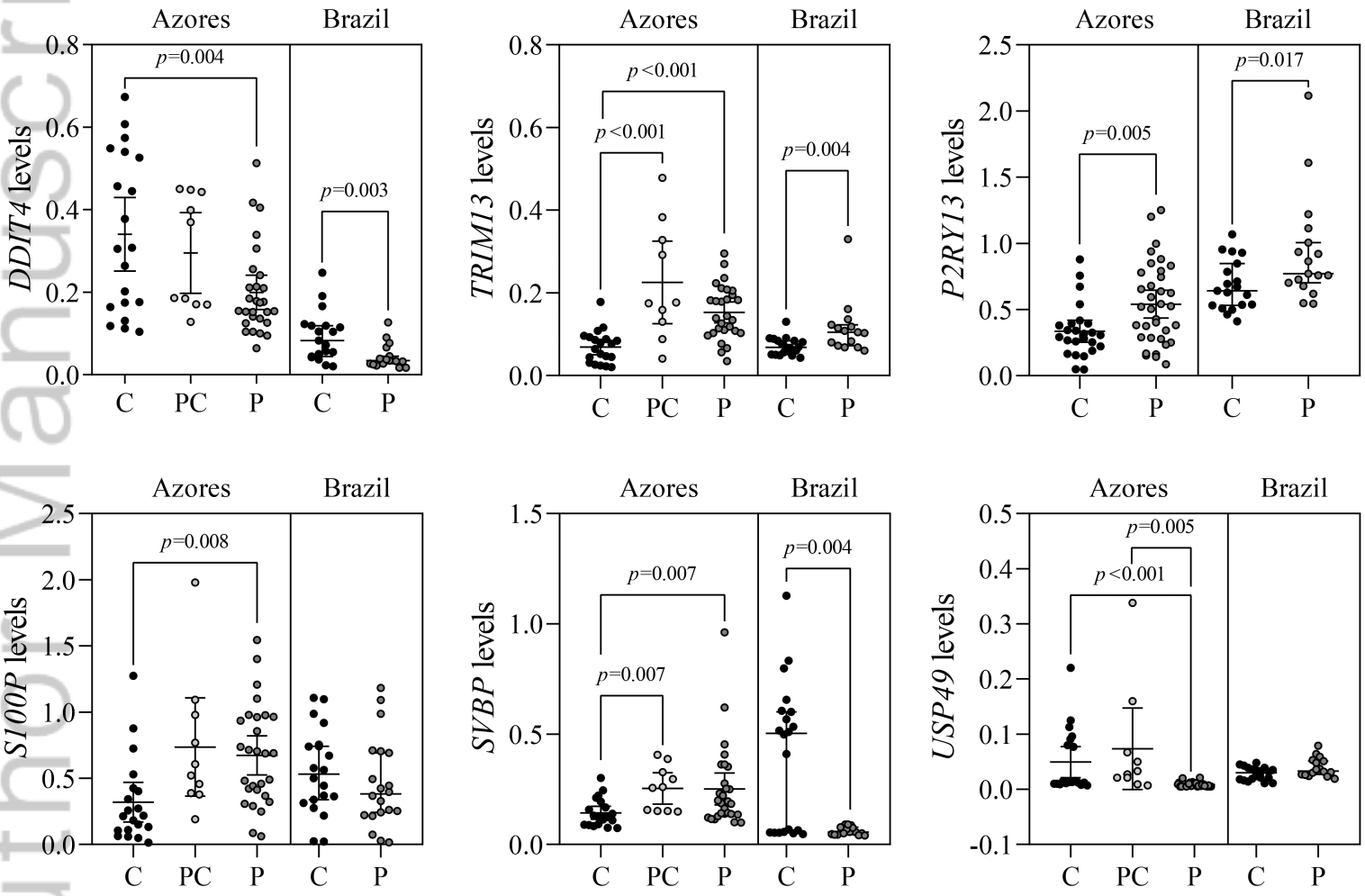


Figure 2.tif

Author Manuscript

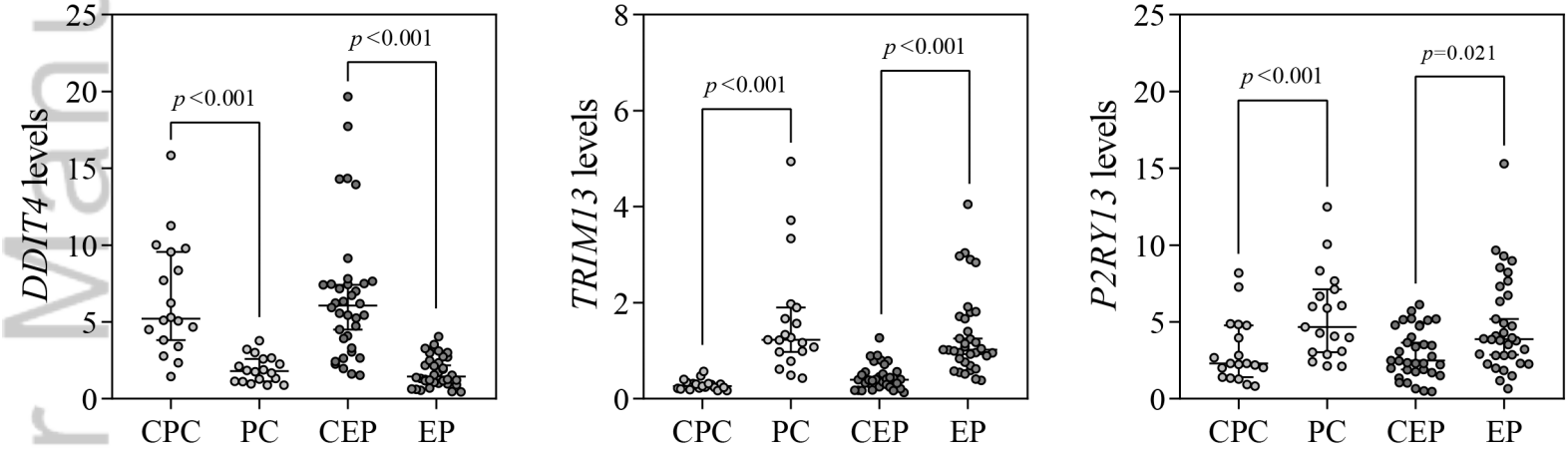


Figure 3.tif

Author Manuscript

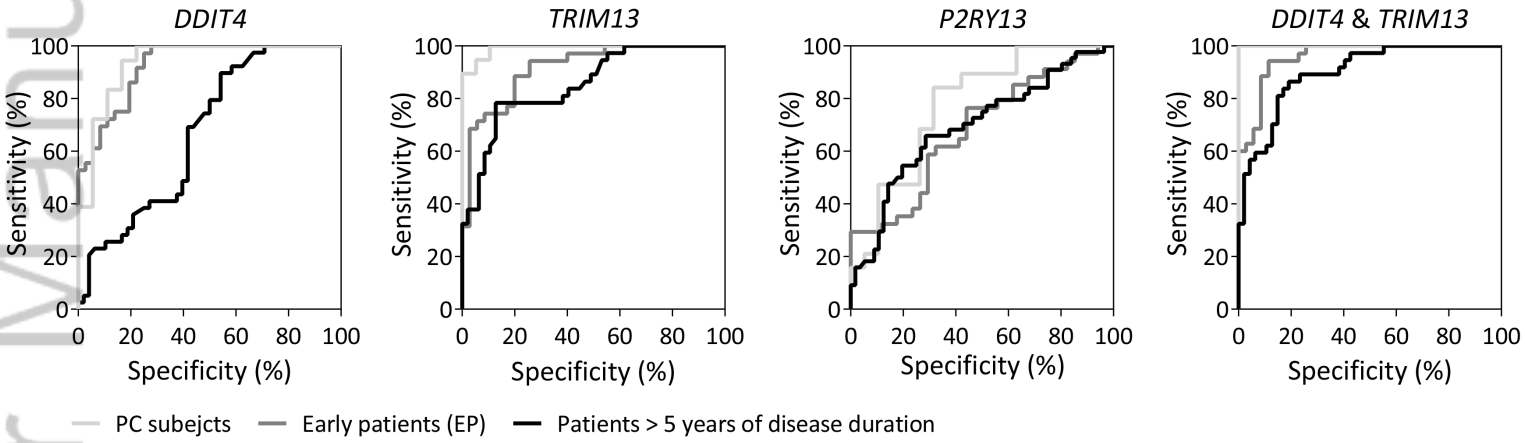


Figure 4.tif

Author Manuscript

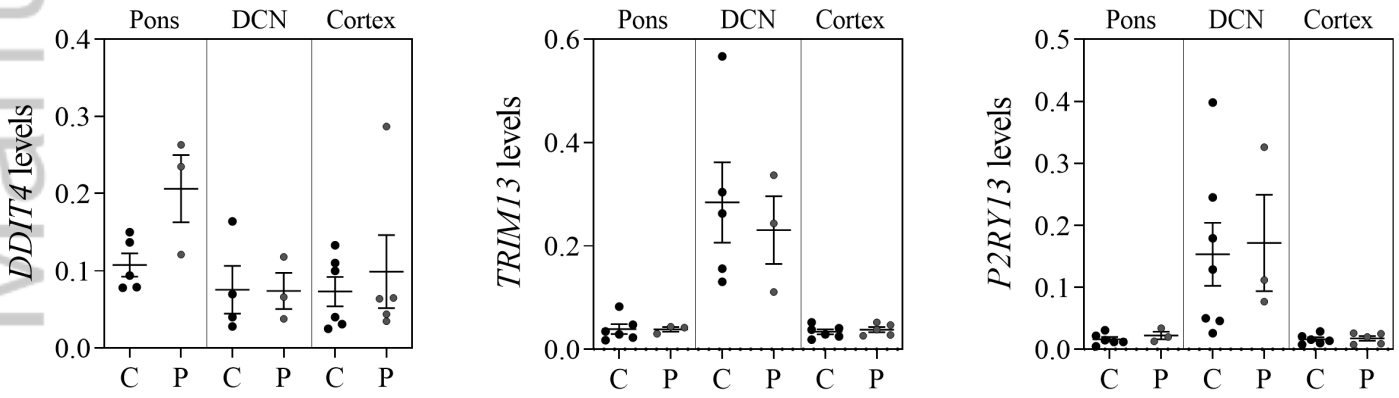


Figure 5.tif

Author Manuscript

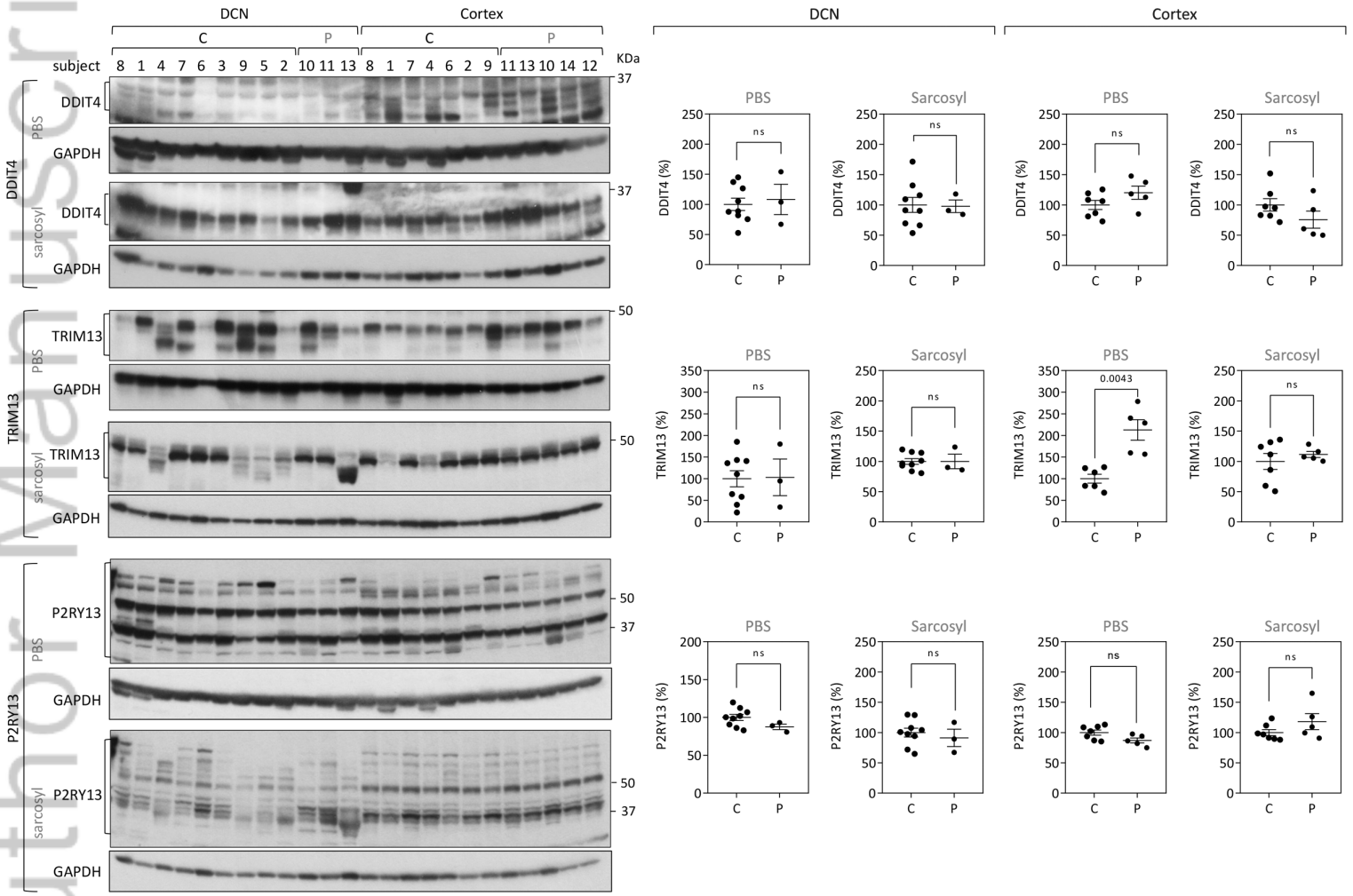


Figure 6.tif