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Customized Dietary Intervention Avoids Unintentional Weight Loss and Modulates Circulating miRNAs Footprint in Huntington's Disease

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Scope: Huntington's disease (HD) is a rare progressive neurodegenerative disorder of genetic origin, with no definitive treatment. Unintentional weight loss (UWL) is a clinical feature of symptomatic HD subjects. To prevent UWL, a customized HD diet is designed and its impact on plasma miRNA HD footprint and neurological parameters is examined.
Methods and results: Eleven participants are included, BMI $\leq$ 18 kg m^{-2} or UWL of 5% in 6 months or 10% in a year. Diet design is based on nutritional surveys and interviews of participants and caregivers and on published literature review. Twelve-month dietary intervention, with follow-up every 3 months, induces high diet adherence, which manages to curb UWL in all participants (73% gained weight). Noticeable increases in fat mass and leptin levels are obtained. The results also show significant decrease in the expression of 19 miRNAs, which are previously reported to be upregulated in HD-patients versus healthy controls: revealing hsa-miR-338-3p, hsa-miR-128-3p, hsa-miR-23a-3p, and hsa-miR-24-3p as potential HD-biomarkers. The diminished expression of hsa-miR-100-5p reflects the general maintenance of the functional status. Cognitive status is improved in six of 11 participants, while only three present better motor-score values.
Conclusion: A customized HD-diet prevents UWL and modified miRNAs HD-footprint. The normalization of miRNA values suggests its potentially use as HD-biomarkers.

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1. Introduction

Huntington's disease (HD) is a rare progressive neurodegenerative disorder of genetic origin, clinically characterized by motor features, psychiatric symptoms, and cognitive decline, caused by an autosomal dominant expanded trinucleotid of CAG-repeat in the huntingtin (*HTT*) gene.[1] It encodes for an abnormally long polyglutamine repeat in the huntingtin protein. Between 36–39 CAG-repeats the disease is manifested with a variable age-dependent penetrance, but above 40 repeats there is a full penetrance.[2] The clinical mean manifest age of onset is 40, with death occurring in 15–20 years. A definitive treatment for HD has yet to emerge.[3] Huntingtin is a very ubiquitous protein highly expressed in the brain and testes.[4] In the central nervous system (CNS), the protein aggregates in the striatal cells, contributing to a neuronal and synaptic dysfunction, which leads to neuronal death and ultimately the signs and symptoms of HD.[5] However, there is an increasing evidence of peripheral abnormalities caused by local expression of mutant huntingtin, which suggests that HD is a systemic disease: glucose intolerance, testicular atrophy, cardiac failure, osteoporosis, gastrointestinal abnormalities, unintentional weight loss (UWL), and muscle wasting.[4,6] Research investigating HD metabolism is often dissimilar due to difficulties in measuring metabolism in humans.[7,8] However, it has recently been reported that circulating microRNAs (miRNAs) may be used as biomarkers in HD.[9] miRNAs are small non-coding RNAs present both in tissues and biological fluids where they mediate the intercellular communication. These miRNAs are upregulated in HD patients, and most of them are involved in cholesterol metabolism, suggesting a potential impairment of this pathway.[9] Circulating miRNAs (c-miRNAs) are used as biomarkers to monitor diseases and the effect of diets.[10] However, whether c-miRNAs footprint of HD might be used to monitor the disease progression remains unknown.[9]

In HD, strength and muscle mass, fatigue, mood, and cognitive function are impaired, and significant correlation has been reported between total motor disability score and body mass index (BMI) and arm muscle circumference.[11] A lower BMI has been associated with a higher rate of disease progression, independently of mutant CAG-repeat size, indicating that other factors must account for this relation.[12] Appropriate nutritional management, following HD genetic confirmation, has been proposed in a set of specific HD-guidelines, focusing on preventing a low BMI.[13,14] However, certain dietary factors could favor the earlier appearance of the symptoms in premanifest HD-patients.[15] An observational study showed that adequate dietary intake prevents against UWL, although it is not associated with better functional state.[16] In this regard and considering the need to initiate a prospective nutritional intervention study in symptomatic patients, a customized HD-diet was designed to determine whether proper feeding is capable of preventing UWL associated with the disease, modifying the miRNAs HD-footprint[17,18] and inducing an improvement in neurological parameters.

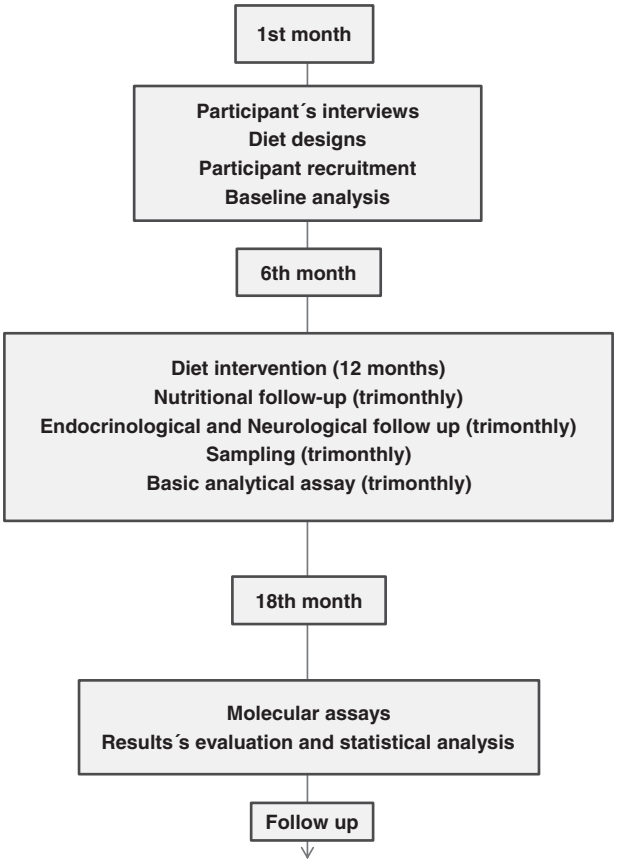

Figure 1. Chronological scheme of the study.

2. Experimental Section

2.1. Recruitment Process

Over a 6-month period, the endocrinology division and neurology department of the Fundación Jiménez Díaz (FJD) Hospital (Madrid, Spain) interviewed HD-patients and their relatives/caregivers to determine nutritional and life habits, difficulties (dysphagia or chewing problems), and also the suitability for the enrollment in the study (**Figure 1**). The main inclusion criteria were BMI $\leq$ 18 kg m^{-2} or UWL of 5% in 6 months or 10% in a year. In addition, possible oral feeding, caregivers and family involvement, and no suspected survival less than 1 year were evaluated. Patients who fulfilled the nutritional parameters were invited to participate (Table S1, Supporting Information). All were diagnosed with manifest HD (>40 CAG-repeats), having a total motor score on the Unified Huntington's Disease Rating Scale (UHDRS-TMS) of >5. Tests are conducted by a trained examiner, rating the main motor symptoms of HD (ocular movements, speech, chorea, and alternating movements).[19] The items were evaluated on a scale ranging from 0 = normal to 4 = severe impairment. Disease severity was defined using a Total Functional Capacity (TFC) score,[19] which was derived from reports of the participant/caregivers and quantified the ability of a participant to perform basic and instrumental activities of daily living. This scale rates the patient's independence and functional capacity according to five items: ability to engage in employment, manage finances, perform domestic chores, personal activities of daily living, and setting for level of care. Each domain ranged from

Table 1. Frequency recommendations for consumption of essential foods.

Frequency	Food	Nutrient
4–5 days per week	Vegetables rich in purines (asparagus, spinach, mushrooms, peas, chard, cauliflower, leeks, truffles or mushrooms)	Purines
3–4 days per week	Shellfish (clams, lobster, oyster, crab, shrimp)	Purines
	Fatty fish (mackerel, sardines, anchovies, ventresca, tuna)	PUFA
2–3 days per week	Nuts (peanuts, walnuts, cashews)	BCAA
	Legumes (lentils, chickpeas, beans)	BCAA
Daily	Fruits and natural juices	Antioxidants
	Salads, vegetables	Antioxidants
	Only 1 dairy product	
	Extra virgin olive oil	MUFA

0 = unable to 2/3 = normal, with a minimum score of 0, and a maximum score of 13 indicating normal functioning.

The TFC score was used to determine the stage of the disease (Stage I: 11–13; Stage II: 7–10; Stage III: 3–6; Stage IV: 1–2; Stage V: 0).[20] Cognitive status was assessed by the mini-mental state examination (MMSE). Duration of the disease was defined as the estimated time elapse since the onset of motor symptoms (Table S1, Supporting Information).

The usual biochemical values assessed in the malnutrition states were within the range of normality, as were those for blood glucose levels, lipid profile, lymphocytes, cholesterol, total proteins, albumin, and electrolytes. A specific diet was prescribed to 36% of patients with dysphagia (without mixing of food textures) and depending on the case, the use of thickeners (NutAvant Espesante C.I.504327) was recommended. Body fat levels measured by body composition were relatively acceptable; however, most of the participants were included due to UWL in recent months (Table S1, Supporting Information).

2.2. Dosage Information

When prescribing the caloric intake for each diet, the aim was to reach between 25 and 35 kcal kg^{-1} d^{-1}, thus covering the appropriate energy requirements.[14] Also, there was a variation of 1800 kcal d^{-1} diet for easy mastication with crushed foods and purees. These diets were complete, balanced, sufficient, varied, and adapted. Additionally, participants were provided with a 400 kcal oral supplement (Nutavant Plus C.I.504746; Table S2, Supporting Information), to maintain an acceptable nutritional status.[21] Moreover, several essential bioactive compounds were included based on their demonstrated benefit in HD due to the quality/quantity of their nutrients (**Table 1**):

1) Foods rich in uric acid (UA) were included due to an association between higher levels of UA and a slower progression of HD, particularly as evidenced by TFC.[22] For this reason, dairy consumption was limited as it was inversely associated with plasma urate concentration.[23]
2) Gamma-aminobutyric acid (GABA) was a neurotransmitter. In humans, GABA was directly responsible for the regulation of muscle tone. Specifically, in HD, the biochemical consequence of the disease was a decrease in GABA. Some foods with a high proportion of essential fatty acids (e.g., MUFA, PUFA) such as olive oil and fish oil, has been shown to prevent the reduction of GABA levels.[24]
3) Cereals, legumes, nuts, meats, eggs, and fish are rich in branched chain amino acids (BCAAs). In HD patients, low plasma values of valine, leucine, and isoleucine were detected, which were related to neurological defects in the brain, muscle tremors, and proteolysis exacerbation.[25]
4) Also, the diet included an increase in the consumption of vegetables, fruits, and juices to elevate the level of antioxidants. In HD, it was reported that antioxidants may exert beneficial effects, promoting the functional neurogenesis. Specifically, dietary polyphenols ameliorated anxiety and depression via regulation of adult hippocampal neurogenesis, while flavanoids protected hippocampal neurons from oxidative stress.[26]

The dossier prepared for dietary counseling included advice on specific foods that were potentially beneficial in HD, templates, predefined menus (Apendix 1, Supporting Information), recipes, and shopping lists in order to facilitate the work of caregivers and family members and also to improve study adherence.

2.3. Nutritional Intervention and Follow-Up

The nutritional intervention was carried out for 1 year (Figure 1). Throughout this period, nutritional/endocrinological/neurological follow-ups were held every 3 months. During the nutritional/endocrinological sessions, the prescribed guidelines were reviewed, patient/caregiver queries were answered, and anthropometric measurements were taken including weight. Nutritional supplementation was adjusted depending on the weight evolution and subjectivity of the relatives/caregivers. Moreover, bioelectrical impedance analysis was performed between 8 and 10 a.m. after an overnight fast using a body composition analyzer (TBF-300 Tanita-Feed; Tanita-Corporation, Japan). In each participant, fat-free mass was then expressed as a percentage of body weight (BW). During the neurological follow-ups the physician performed UHDRS-TMS, TFC, and MMSE.

Each participant at starting point ($t = 0$) played the role of reference value to perform a comparison with values recorded at 3, 6, 9, and 12 months.

2.4. Samples

Blood samples were collected on each visit to the hospital, after an overnight fasting.

1) Plasma extraction (EDTA-tube): samples were centrifuged at $1300 \times g$ during 15 min and stored at −80 °C until processing.
2) DNA isolation: SpeedTools DNA Extraction Kit (Biotools, Spain) was used, and DNA was stored at −80 °C until assaying.

Independently, blood samples ($t = 0$ and $t = 1$ year) of five genetic diagnosed HD-subjects with significant weight loss and without participation in the diet intervention program but,

integrated in the follow-up agenda of the Department of Neurology at FJD Hospital were obtained for comparative purposes (Table S3, Supporting Information).

The clinical information and samples given were collected through informed consent and meeting the requirements of the Declaration of Helsinki (n° 01/16 -Jan 12 2016-).

2.5. Assays

2.5.1. Biochemical Parameters

Fasting glucose, insulin and HbA1c, total cholesterol, HDL-C, LDL-C, triglycerides, lymphocytes, serum albumin, total protein, sodium, and potassium values were measured by the clinical biochemistry unit of the FJD Hospital.

2.5.2. Genetic Profile

The DNA samples of the 11 participants were analyzed to determinate of the number of CAG-repeats following the preferred method of the American College of Medical Genetics and Genomics committee for HD genetic testing.[27]

2.5.3. Determination of Blood Leptin Concentrations

Plasma leptin was measured with commercial ELISA from BioVendor (RD191001100 Human-Leptin-ELISA) according to the manufacturer's instructions. As the limit of detection of the assay was 0.2 ng mL^{-1}; values below that limit were set to 0.2 ng mL^{-1}.

2.5.4. miRNA Determination

Total RNA from plasma was isolated using miRCURY RNA isolation kit (Exiqon, Denmark) and cDNA was synthesized using miScript II- Reverse transcription kit (Quiagen, Germany) following the manufacturer instructions. Twenty-five relevant miRNAs in HD were analyzed by quantitative real time PCR (qRT-PCR) as previously described.[9] The c-miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5.5. Bioinformatic Analysis

To predict the possible tissue of origin of the miRNAs, hierarchical clustering analysis was performed using the Human miRNA TissueAtlas (1.0) database.[28] Validated target genes of differentially expressed miRNAs supported by strong experimental evidence were obtained from the miRWalk 2.0 database. Functional enrichment of target genes was performed with the GeneCodis3 algorithm using Gene Ontology (GO) and Panther Pathways annotation.

For gene interaction (GI) analysis, a subset of miRNAs was selected from those that were significantly changed by dietary intervention in HD-patients. A total of 19 miRNAs were analyzed using the above-mentioned target genes. The interaction network between the gene targets and modulated miRNAs was performed as previously described,[29] using the above-mentioned target genes targeted by at least two miRNAs. Target dot sizes were directly correlated with the number of interactions with the miRNA's set.

2.5.6. Statistical Study

Results are expressed as mean ± SEM. The statistical significance ($p < 0.05$) of the increments was assessed by one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. According to Levene's test, all variances were homogeneous. Analyses was performed using SPSS V21(IBM, NY, USA). The Kolmogorov–Smirnov test was used to analyze the normality of the variables studied. Determination of statistical significance of the miRNA modulation ($t = 0$ and 12 months of dietary intervention) was performed using *t*-test analysis paired samples, using Graph pad Prism V.5 (La Jolla, CA, USA).

3. Results and Discussion

3.1. Nutritional Intake

Overall, preintervention, detailed interviews elaborated by the nutritionist (Appendix 2, Supporting Information) revealed that patients had a low-to-moderate intake of macronutrients, thus coinciding with a 1989 study that reported a low fat and carbohydrates intake in HD-patients.[30] After an exhaustive review of guidelines and literature, the nutritionist followed the recommendation of 25–35 kcal kg^{-1} d^{-1},[14] deciding against the high calorie intake frequently recommended (3500–5000 kcal d^{-1}), and including essential bioactive compounds.[22–26] On that basis, a customized diet was designed for each participant, and adherence to these recommendations was high, with the exception of participant number 11, who was diagnosed with anorexia nervosa. Given the individual patient characteristics, most participants reached 1750–1800 kcal d^{-1}. Despite the attempt to facilitate an adequate intake with the use of enteral nutrition, 27% of the participants had an intake that was lower than the one prescribed. Aiming to facilitate the achievement of 35 kcal kg^{-1} d^{-1}, enteral supplementation was provided by the nutritionist (**Table 2**).

3.2. Body Weight and Composition

In this study, after a year of nutritional intervention, eight of 11 participants showed an increase in BW, while a very slight decrease was observed in the other three (**Table 3**). Specifically, six patients gained between 0.8 and 1.6 kg, and two individuals increased 4.1 and 8.5 kg, respectively. In contrast, three subjects diminished the BW on a small scale (0.3–0.7 kg). The intervention clearly curbed UWL in all participants. Interestingly, in most of cases (73%) the diet even induced weight gain. This result was relevant due to the fact that in HD, UWL was a chronic symptom even despite an adequate caloric intake. Increased total energy expenditure with higher basal resting energy has previously

Table 2. Nutritional intake.

HD subject	1	2	3	4	5	6	7	8	9	10	11
Diet (kcal)	1500	1800	1800	1750	1800	2000	1750	1500	1750	1800	1500
Nutritional supplies (kcal)	≈400	≈400	≈800	≈400	≈400	≈800	≈400	≈400	≈800	≈1200	≈800
Prescribed intake (kcal)	1900	2200	2600	2150	2200	2800	2150	1900	2550	3000	2400
Performed intake (kcal)	1900	1800	1900	2150	2150	2800	2150	1900	2550	3000	300[a]
Dysphagia			Yes		Yes			Yes		Yes	

[a] Participant 11 was diagnosed with anorexia nervosa.

Table 3. Body weight and composition.

HD subject	1	2	3	4	5	6	7	8	9	10[a]	11[a]
Weight (kg)											
Starting point	56.2	83.9	59.6	61.7	70.0	73.6	62.6	55.2	56.7	71.2	46.0
3 months	57.8	81.7	61.5	62.8	75.3	73.6	64.4	54.1	57.7	70.5	44.7
6 months	57.5	83.7	60.5	65.5	80.0	71.8	66.4	56.3	58	70.5	45.3
9 months	56.6	85.0	61.6	65.0	81.4	76.6	66.0	56.8	58.4	70.5	43.5
12 months	57.8	83.6	63.7	62.7	78.4	74.9	63.9	54.5	57.5	72.4	45.4
Fat (kg)											
Starting point	15.2	22.7	19.7	18.3	15.5	16.7	11.3	6.5	6.7		3.3
3 months	15.1	19.6	19.3	19.0	16.9	15.3	12.0	13.2	7.3		
6 months	15.8	22.5	20.5	22.5	22.2	17.3	13.4	15.5	7.3		
9 months	14.7	24.7	20.9	18.3	24.8	19.2	13.7	15.2	8.2		
12 months	16.5	23.1	23.1	21.5	20	17.6	11.4	15.6	7.4		
Free-fat mass (kg)											
Starting point	41.0	61.2	39.9	43.4	57.3	56.9	51.3	48.7	50.0		42.0
3 months	42.7	62.1	42.2	43.8	58.4	56.5	52.6	40.9	50.4		
6 months	41.7	61.2	40.0	43.0	57.8	57.3	53.0	40.8	50.7		
9 months	41.9	60.3	40.7	43.4	56.6	57.4	52.3	41.6	50.2		
12 months	41.3	60.5	40.6	41.2	58.4	57.3	52.5	40.5	49.8		
Water (kg)											
Starting point	30.0	44.8	29.2	31.8	42.3	41.7	37.6	35.7	36.6		30.7
3 months	31.3	45.5	30.9	32.1	42.8	41.0	38.5	20.9	36.9		
6 months	30.5	44.8	29.3	31.5	42.3	41.9	38.8	29.9	37.1		
9 months	30.7	44.1	29.8	31.8	41.4	42.0	38.3	30.5	36.8		
12 months	30.2	44.3	29.7	30.2	42.8	41.9	38.4	30.6	36.5		—

[a] Values for patients 10 and 11 were not available. The former showed a high level of choreic movements, which made accurate measurement difficult, while the latter did not reach the minimum detectable values in equipment.

been reported in symptomatic HD patients.[31,32] Furthermore, weight loss has been also observed in presymptomatic HD mutation carriers, with a higher caloric intake than that of healthy controls.[33] It therefore clears that nutritional monitoring is essential to HD management.

Patients with a high BMI at baseline exhibited a slower rate of HD progression in all clinical domains including functional capacity, motor function, and cognitive performance.[12] However, it should be noticed that a higher caloric intake is a potential risk factor for earlier initiation of the symptoms in asymptomatic patients.[15]

An observational multicenter Spanish dietary intake study reported that in advanced HD patients, an adequate dietary intake protected against UWL, although this intake was not associated with better neurofunctional state.[16] However, in HD-moderate patients, who self-reported dietary intake using a 3-day dietary record, an association was found between Mediterranean diet adherence, and quality of life, lower comorbidity, and motor impairment compared to those participants with low adherence.[34]

In our study, body composition was measured in nine of 11 participants, all of whom showed an increase in fat mass. Four had a mass increase of between 0.1 and 0.9 kg, and in the other five between 1.3 and 9.1 kg. Regarding the fat-free mass, four participants had a slight increase and the rest showed a reduction that mainly correlated with lower body water content (Table 3). The main changes in body composition were an increase in fat tissue and, therefore, a reduction (not in all) of the nonfat tissue, corresponding mostly to body water reduction but not to muscle mass. The analytical assays did not show significant differences before and after a year of nutritional intervention (data not

show). However, evaluation of the levels of leptin demonstrated that after 12 months of dietary intervention, the hormone was increased in seven participants ($\Delta = 1.30/12.07$ ng μL^{-1}]; the values were decreased in one [$\Delta = -1.04$], no change was observed in two [−0.48, 0.44], and patient number 11 had leptin values that fell below the limit of detection (Figure S1A, Supporting Information). Data referring to leptin values in five HD subjects, not receiving nutritional advice for 1 year and with significantly UWL (3.9 ± 0.8 kg), revealed reduced levels in four cases ($\Delta = -2.77/-1.87$; $p < 0.05$), while no change was observed in a patient (0.28; Figure S1B, Supporting Information). Although some authors indicated that there was no relationship between leptin production and body fat mass levels,[35] in this study, we found that patients with a higher level of fat tissue, who followed the nutritional intervention, showed increased levels of leptin, unlike HD-patients not receiving a customized diet. These data confirmed that leptin values acted as a valuable predictor of the absence of fat mass in HD.[36]

3.3. microRNAs

Twenty five c-miRNAs reported to be altered in HD or associated to clinical outcomes[9] were examined in response to the 1-year dietary intervention. The c-miRNAs expression was monitored every 3 months (Figure S2, Supporting Information). Interestingly, most of the miRNAs that were upregulated in HD patients compared with healthy controls,[9] showed decreased expression beyond the Bonferroni correction *p*-value threshold of 0.05 (**Figure 2**). Considering c-miRNAs affected by disease stage, the outcome was quite heterogeneous. The disease parameter TFC was associated with variations in hsa-miR-100-5p, hsa-miR-641, and hsa-miR-330-5p levels, while hsa-miR-122-5p was related to scores on the UHDRS scale.[9] It was clear that the expression of hsa-miR-100-5p was significantly and favorably diminished (Figure 2). However, multivariate logistic regression pointed out that there was no association between modification of hsa-miR-100-5p, hsa-miR-641, hsa-miR-330-5p, or hsa-miR-122-5p and parameters such as age, sex, number of CAG-repeats, disease onset, or weight modification. This result may become clearer with an increase in the sample size.

Those c-miRNAs which significantly changed, were analyzed in five HD subjects with similar number of CAG-repeats and clinical characteristics to those included in the study, but not receiving nutritional counseling and with UWL in one year. None of the significant c-miRNAs reduced by the diet were modulated in the untreated group (**Figure 3**). These relevant results established a significant modulation of the cmiRNA profile by the dietary intervention, trending to normalization of miRNA values.

3.4. Target Genes and Predictive Functionality of the Modulated miRNAs

In silico analysis of the possible tissue of origin and hierarchical clustering analysis were performed with the c-miRNAs significantly modulated by the dietary intervention. Information related to hsa-miR-223-5p or hsa-miR-942-5p was not available in the TissueAtlas database (**Figure 4**). Based on the results, two regions were found to be noteworthy. At the bottom, hsa-miR-338-3p and hsa-miR-128-3p were markedly and selectively enriched in brain structures and the spinal cord. Altered expression of both was previously reported in the frontal cortex and striatum of HD-patients. Specifically, hsa-miR-338-3p showed upregulated levels, in agreement with that detected in plasma. However, has-miR-128 in the striatum of HD-subjects and mouse HD-model had reduced expression, opposite to that obtained in plasma, where it was upregulated.[9,37,38]

Interestingly, hsa-miR-338-3p, which was found in the non-coding intron region of the of the apoptosis-associated tyrosine kinase (AATK) host gene, was associated with apoptosis when upregulated.[39] Moreover, it played a role in mitochondrial function, being enriched in the distal axons and having an impact on oxygen-dependent metabolic pathways in neurons that regulated cytochrome c in the IV subunit.[40,41]

Regarding hsa-miR-128, its overexpression was related to decreases of BAX gene/protein levels and increases in the apoptotic activity.[42,43] In neurodegenerative diseases, when the mitochondrial complex I presented disrepair on mitochondrial respiration, an activation of cell death occurred, depending on the BAX gene, due to oxidative stress.[44] At the top of Figure 4, homogenous expression areas for hsa-miR-23a-3p and hsa-miR-24-3p were identified. Beside their enhancement in the brain,[38] both were evenly more enriched in CNS, bone, muscle, myocardium, bladder, skin, epididymis, thyroid, esophagus, and colon. These two miRNAs were previously described as almost significantly upregulated in HD plasma compared to healthy subjects, although upregulated expression of hsa-mir-23a-3p was reported in the frontal cortex and striatum from HD-patients.[9,38] By taking into account the role of hsa-miR-338-3p and hsa-miR-128 in neuronal function, their tracking in peripheral fluids, and also the potential modulation by the diet, both seem to be suitable biomarkers for HD. In addition, hsa-miR-23a-3p and hsa-miR-24-3p could be useful for monitoring of disease aspects also beyond brain tissue.

From the analysis of 19 c-mirRNAs by means of miRWalk (2.0), which were significantly modified by the dietary intervention, the results showed that all of these c-miRNAs presented validated targets supported by strong experimental evidences. GO analysis of those target genes suggested that the following were the main biological processes involved: response to stress (GO:0006950), regulation of phosphate metabolic process (GO:0019220), or immune system process (GO:0002376). Moreover, data obtained from Panther Pathways analysis suggested the involvement in interleukin signaling pathway (P00036), inflammation mediated by chemokine and cytokine signaling pathway (P00031), or apoptosis signaling pathway (P00006) (Table S4, Supporting Information).

An important feature of miRNAs is that one gene can be regulated by different miRNAs. To search for possible validated target genes potentially modulated by more than one miRNA and which responded to the dietary intervention in HD-patients, GI analysis was performed (**Figure 5**).[29] Putative GIs (number of dietary HD-modulated miRNAs that can potentially target the 3′UTR of the protein coding gene showed) were obtained using validated target genes from databases (see Experimental Section). Only genes targeted by three or more miRNAs are depicted. Several genes were

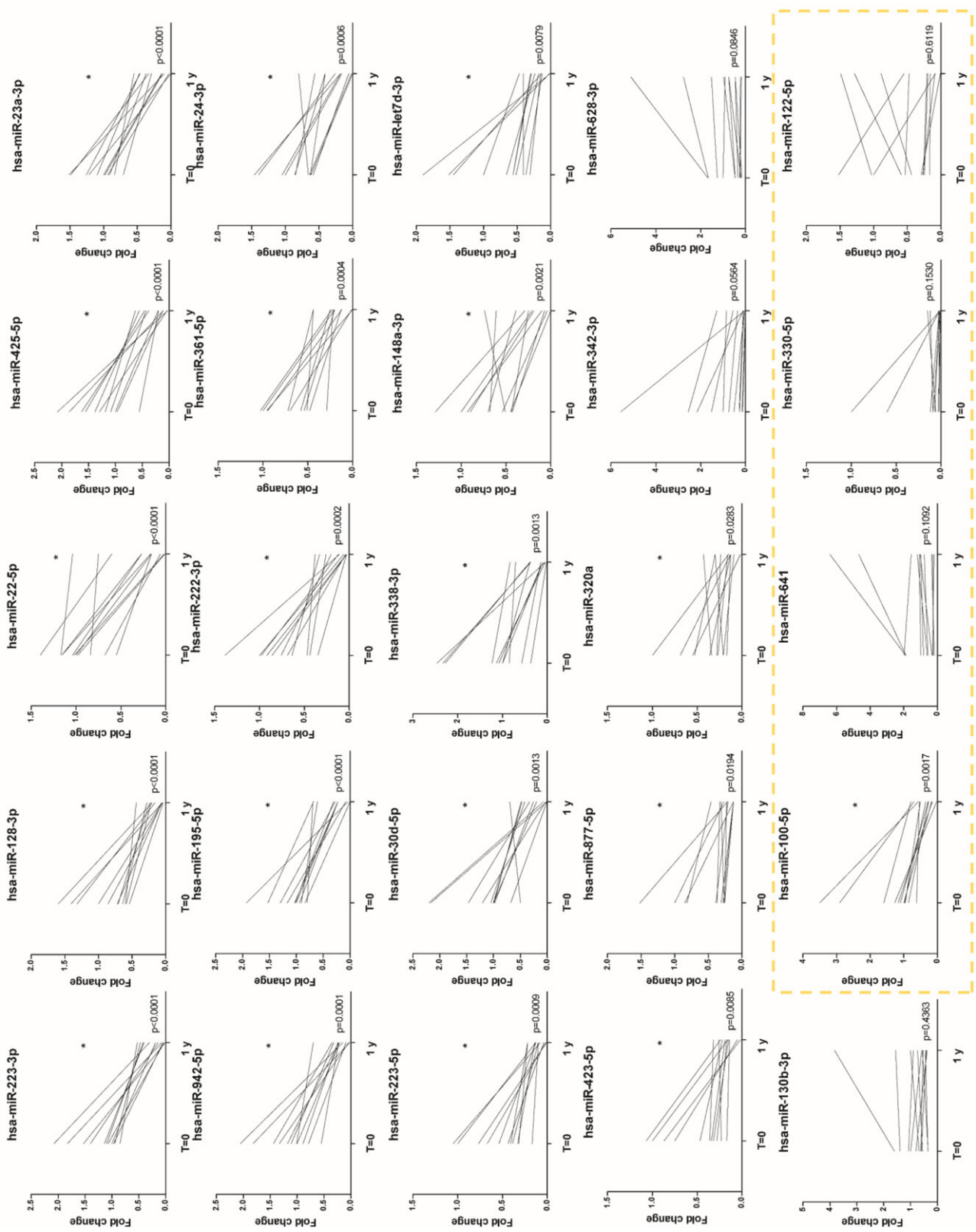

Figure 2. c-miRNA expression profiles of HD patients ($n = 11$) undergoing a 1-year dietary intervention. The yellow square indicates the four c-miRNAs specifically related to HD clinical outcomes.

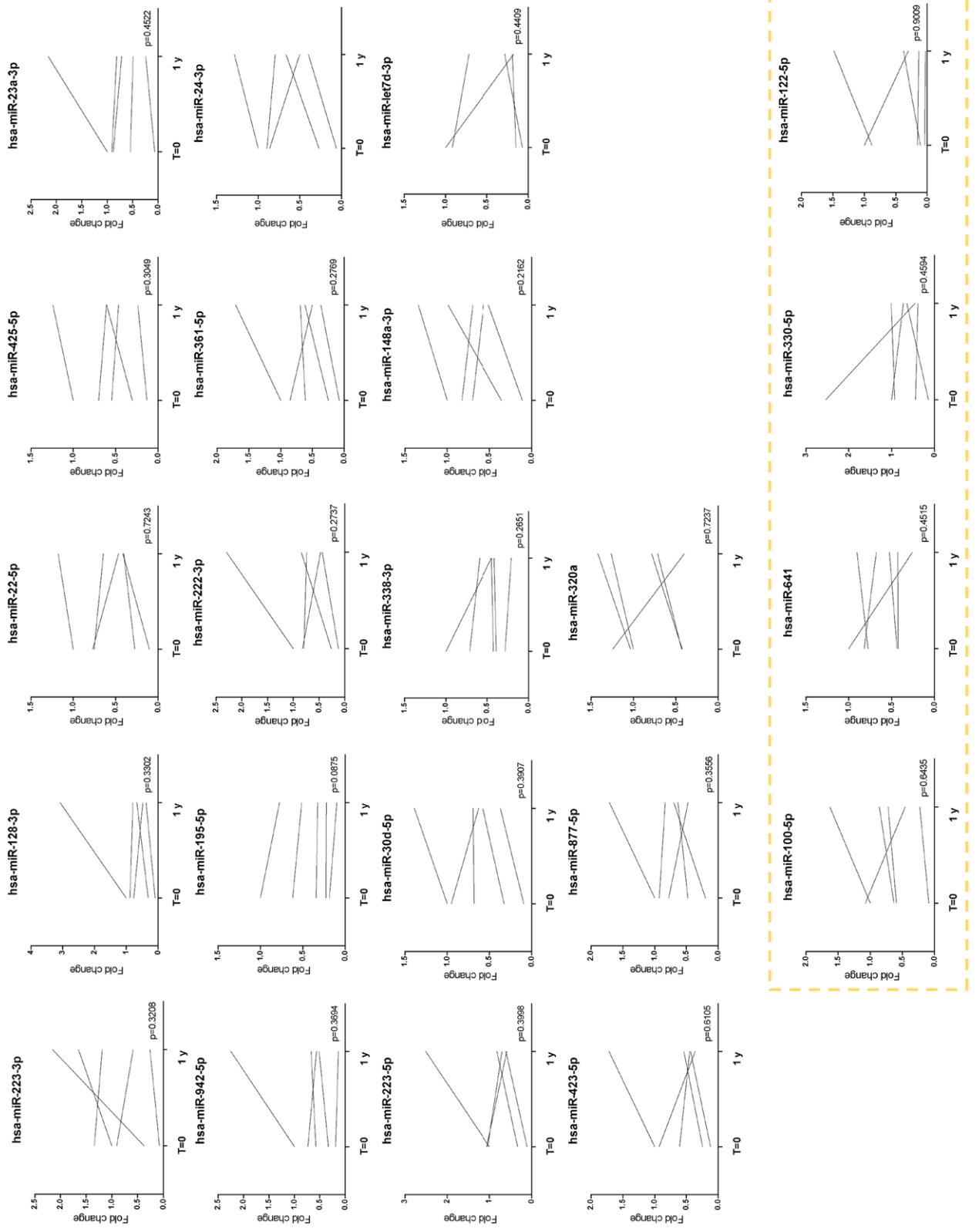

Figure 3. c-miRNA expression profiles of HD patients ($n = 5$) not undergoing nutritional councelling over a 1-year period. The analysis was restricted to those c-miRNAs significantly changed by the dietary intervention plus four miRNAs specifically related to HD clinical outcomes (yellow frame).

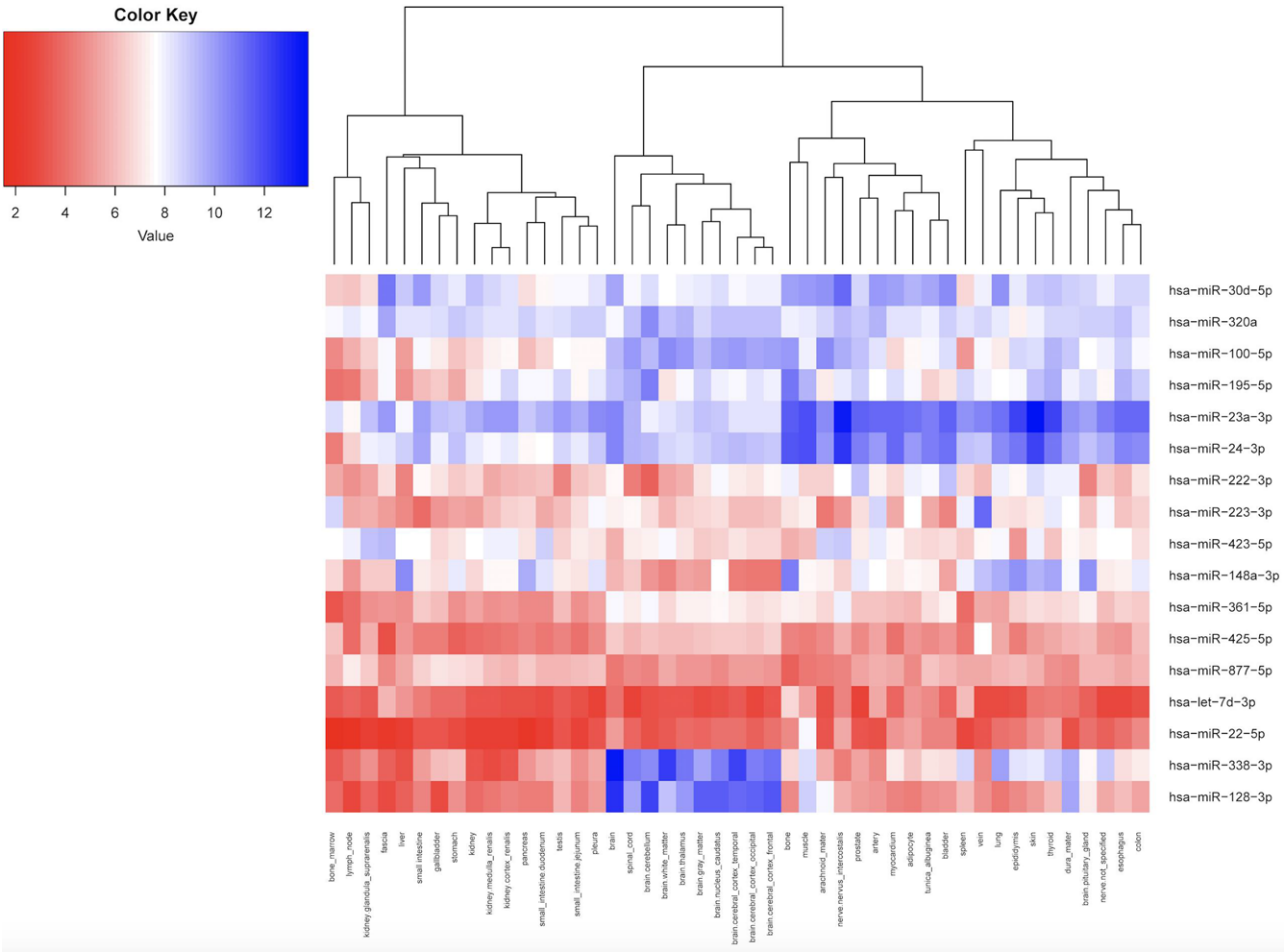

Figure 4. *In silico* analysis of the possible tissue of origin of the c-miRNAs significantly modulated by the dietary intervention and hierarchical clustering analysis. The TissueAtlas database did not provide information related to hsa-miR-223-5p or hsa-miR-942-5p.

predicted to be regulated by more than three modulated miRNAs. Interestingly, key genes involved in apoptosis, PDGF/IGF, or the angiogenesis pathway showed higher GIs (*Pten, Runx2, Foxo3, Tp53, Myc, Cdkn1b, Igf1r, Notch1,* and *Vegfa*). Other genes having more than five GIs included *Fbxw7, Tmed7, Chuk*, and *Mafb*. The literature describes that several of these genes (i.e., *Fxo3, Tp53*, and *Vegfa*) are involved in HD.[45–47] Whether the selected miRNAs described here were solely responsible for gene regulation remains to be determined and deserves further research.

3.5. Neurological Parameters

3.5.1. Disease severity

According to the TFC scale, most of the patients maintained their functional status during the year of follow-up. In particular, three patients were enrolled in stage I of the disease and this situation remained unchanged, four were enrolled in stage II, and just one worsened to stage III. There were two other patients who started the study in stage III and maintained their functional stage. Finally, two patients were in stages IV and V, and they equally maintained the score in the observational neurological study (Table S5, Supporting Information).

3.5.2. Cognitive status

Most of the patients (6/11) improved their cognitive status according to the MMSE and four experienced just a slightly worsening. Patient number 4 showed a significant worsening, which was also evidenced by the other two neurological parameters; this fact maybe linked to personal and family circumstances (Table S5, Supporting Information).

3.5.3. Motor scores

Only three of 11 patients improved their motor score according to the UHDRS parameters (Table S5, Supporting Information). The diminished expression of hsa-miR-100-5p matched the general

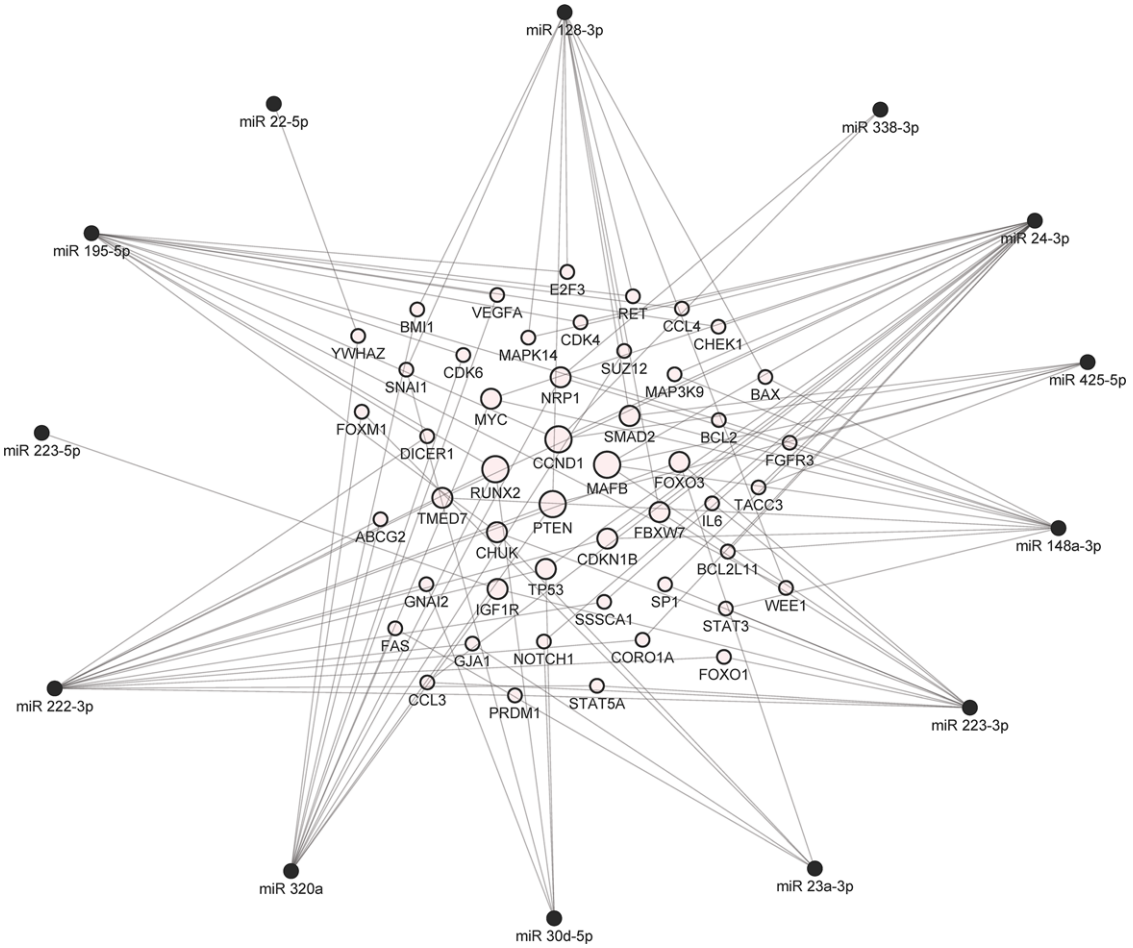

Figure 5. Gene interaction analysis for possible validated target genes potentially modulated by more than one miRNA that responded to the dietary intervention in HD-patients.

maintenance reported in the HD-patient functional status, worsened in four of five HD patients without dietary intervention and UWL. This maybe considered a beneficial effect of the diet on neurological parameters. Beyond this, however, the neurological interpretation was limited. Data were less robust and conclusive than nutritional and molecular results, and definitely more difficult to measure. The small size and heterogenous sample, due to the condition of rare disease, and also the currently available cognitive scales—with needs of further validation in HD—have restricted the neurological results of this study.[48]

3.6. General Considerations

- Preintervention, as a general rule, patients/caregivers reported a high-to-normal intake of calories and also healthy nutritional habits. However, 24-h-recall method clearly revealed a misperception of the real intake of calories, being lower than that reported.
- The aptitude of the patient and caregiver involvement were crucial in the adherence to the study.
- Personal issues along the study showed high impact on neurological scales (i.e., death in the family or geographic location due to trips to Madrid for the visits).

This study confirmed that the environment of the patient played a key role in the disease progression.

4. Concluding Remarks

This study reports that a customized HD diet prevented UWL. Moreover, the dietary intervention significantly modified the miRNA footprint of the disorder. The normalization of miRNA values points out its potentially use as HD biomarkers. The work also suggests several target genes for future therapeutic interventions.

Despite the fact, the results are subjected to further studies with larger and more homogenic cohorts (in number of individuals and controls) extended timeline and validated rating scales to evaluate the nutritional, molecular, and neurological outcomes, it has been demonstrated that timely recognition of UWL and a

multidisciplinary approach could eventually result in considerable improvements, in the quality of life of HD patients.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

diet, Huntington's disease, microRNA, neurology, nutrition

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