RESEARCH

Differences in circulating microRNA signature in Prader–Willi syndrome and non-syndromic obesity

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Abstract

Prader–Willi syndrome (PWS) represents the most common genetic-derived obesity disorder caused by the loss of expression of genes located on the paternal chromosome 15q11.2-q13. The PWS phenotype shows peculiar physical, endocrine and metabolic characteristics compared to those observed in non-syndromic essential obesity. Since miRNAs have now a well-established role in many molecular pathways, including regulatory networks related to obesity, this pilot study was aimed to characterize the expression of circulating miRNAs in PWS compared to essential obesity. The circulating miRNome of 10 PWS and 10 obese subjects, adequately matched for age, BMI and sex, was profiled throughout Genechip miRNA 4.0 microarray analysis. We identified 362 out of 2578 mature miRNAs to be expressed in serum of the studied population. The circulating miRNA signature significantly characterising the two populations include 34 differently expressed RNAs. Among them, miR-24-3p, miR-122 and miR-23a-3p highly differ between the two groups with a FC >10 in obese compared to PWS. In the obese subjects, miR-7107-5p, miR-6880-3p, miR-6793-3p and miR-4258 were associated to the presence of steatosis. A different signature of miRNAs significantly distinguished PWS with steatosis from PWS without steatosis, involving miR-619-5p, miR-4507, miR-4656, miR-7847-3p and miR-6782-5p. The miRNA target GO enrichment analysis showed the different pathway involved in these two different forms of obesity. Although the rarity of PWS actually represents a limitation to the availability of large series, the present study provides novel hints on the molecular pathogenesis of syndromic and non-syndromic obesity.

Key Words

▶ microRNA
▶ miRNA
▶ Prader–Willi syndrome
▶ array
▶ obese
▶ non-syndromic obesity
▶ serum miRNA

Introduction

Prader–Willi syndrome (PWS) is a complex multisystem disorder due to the lack of expression of genes located on the paternal chromosome 15q11.2-q13. Together with Angelman syndrome, a totally different clinical condition,

PWS is the first example in humans of genetic imprinting (1). Three main genetic subtypes are involved namely interstitial deletion of the proximal long arm of paternal chromosome 15 (del15q11.2-q13) (DEL15, 65–75% of

cases), maternal uniparental disomy of chromosome 15 (UPD15, 20–30% of cases) and imprinting defects (IC, 1–3%) (2).

PWS has an estimated incidence rate of 1 in 15,000 live births (3), representing the most common syndromic form of life-threatening obesity. The main characteristics of PWS include infantile hypotonia with feeding problems, early development of severe hyperphagia with food-seeking behaviour, mental retardation, behavioural problems, dysmorphic features and multiple hormonal abnormalities (4). A complex hypothalamic dysregulation is currently thought to lie at the root of PWS phenotype, including the lack of satiety, which provokes compulsive appetite resulting in extreme obesity, if uncontrolled (5). Obesity phenotype of PWS, however, shows physical, endocrine and metabolic characteristics different from those observed in simple obesity. For the same degree of weight excess (6), PWS harbours higher fat mass percentage than non-syndromic obesity, typically affecting abdomen, buttocks and thighs (4). In addition, fat free mass is significantly reduced, negatively affecting skeletal muscle performances (7). Interestingly, PWS is characterised by reduced visceral fat deposits and a predominant accumulation of subcutaneous adipose tissue, in comparison to subjects with common obesity (8). Accordingly to this peculiar body composition, individuals with PWS are characterised by lower insulin levels and higher insulin sensitivity as opposed to obese controls (9). Regardless of their metabolic advantages, however, PWS patients are at risk for developing the comorbidities conventionally related to obesity, including type 2 diabetes mellitus, metabolic syndrome, hepatobiliary complications, cardiovascular and respiratory problems. Overall, PWS subjects show a high prevalence of altered glucose metabolism that appears more common in obese and adult subjects (10), while the prevalence of metabolic syndrome is comparable to that reported in obese controls (11). Conversely, a lower risk of developing severe non-alcoholic fatty liver disease, both in children and adults, has been found in PWS (12, 13).

In recent years, growing evidences have identified miRNAs as important regulators of cellular pathways. They may affect lipid metabolism (14), adipocyte formation (15), metabolic activity (16) and many other pathways in obesity-related disorders. More important, the presence of circulating miRNAs has fostered intriguing studies on the role of those miRNAs as molecular biomarkers. Since miRNAs are actively released from cells into biofluids, they are considered as an important source of information for the characterisation of body diseases. miRNome profile studies may serve as tools for the identification of the molecular alterations distinctive for each pathological condition.

Based on these considerations, the primary aim of this pilot study was to characterise the expression of circulating miRNAs in a genetic-derived obesity disorder, such as PWS, compared to non-syndromic obesity.

Patients

Ten Caucasian patients with PWS (four males, six females, aged 22 to 47 years, mean±s.d. BMI: 37.3±6.8) participated in the study (Table 1). All subjects showed the typical PWS clinical phenotype (4). Genetic analysis was performed and DEL15 was found in all patients. The patients were consecutively recruited from the Division of Auxology, Istituto Auxologico Italiano, Piancavallo (VB), Italy. Four of six PWS women had primary amenorrhoea, four females were undergoing sex steroid replacement treatment. At the time of the study, three individuals were on growth hormone treatment, whereas two had been treated in the past. Five PWS had never received growth hormone therapy.

Age, BMI, sex-matched adult patients (no.: ten, four males, six females, aged 24–44 years) suffering from essential obesity (mean±s.d. BMI: 42.3±5.9), recruited at the Division of Metabolic Diseases, Istituto Istituto Auxologico Italiano, Piancavallo (VB), Italy, were also included in the study. All females had regular menses (three were on oestrogen treatment for contraception). Apart from severe obesity, no overt endocrine disorders (diabetes mellitus, hypothyroidism, hypercortisolism, etc.) were present.

Physical examination included determination of height and weight in fasting conditions and after voiding. Standing height was determined by a wall-mounted Harpenden Stadiometer (Holtain Limited, Crymych, Dyfed, UK). Body weight was measured with subjects in minimal clothes to the nearest 0.1 kg, using standard equipment. BMI was defined as weight in kilograms divided by the square of height in meters. Obesity was defined for any BMI over 30 kg/m^2.

The study was approved by the Human Research Ethics Committee of Istituto Auxologico Italiano, IRCCS, Milan, Italy (ref. no. 01C628; acronym: NAFLDmiRNAPWS). Before the study began, the purpose and objectives had been carefully explained to each patient. Written informed consent was obtained from all participants and their parents (when appropriate).

Table 1 Clinical characteristics of PWS and OB subjects.

Patient ID	Karyotype	Sex	Age (years)	Height (m)	Weight (kg)	BMI (kg/m²)	Waist circ.	Hip circ. (cm)	WHR	Fat mass[a] (%)	Fat free mass[a] (%)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Liver steatosis (degree)	Primary amenorrhea	Sex steroid therapy	Current GH therapy	Previous GH therapy
1PWS	del15	M	42.4	1.51	90.8	39.9	147	123	1.20	38.5	61.5	120	80	No	–	No	Yes	No
2PWS	del15	M	29.1	1.55	69.7	29.1	104	103	1.01	41.8	58.2	120	70	Yes (1)	–	No	No	No
3PWS	del15	M	42.8	1.57	76.4	30.9	103	104	0.99	30.2	69.8	120	80	No	–	No	Yes	No
4PWS	del15	M	24.8	1.44	63.2	30.4	102	102	1.00	44.6	55.4	120	80	Yes (2)	–	No	No	No
5PWS	del15	F	47.2	1.41	91.7	46.1	121	124	0.98	56.6	43.4	130	80	Yes (1)	No	No	No	No
6PWS	del15	F	30.9	1.44	80.6	39.1	106	120	0.88	52.8	47.2	130	80	No	Yes	Yes	No	No
7PWS	del15	F	22.2	1.65	89.2	32.8	112	117	0.96	44.3	55.7	120	80	No	Yes	Yes	Yes	NO
8PWS	del15	F	35.3	1.66	92.0	33.5	106	118	0.90	46.2	53.8	130	80	Yes (1)	Yes	Yes	No	Yes
9PWS	del15	F	45.9	1.43	97.0	47.4	134	130	1.03	56.9	43.1	120	70	Yes (1)	No	No	No	No
10PWS	del15	F	36.75	1.58	108	43.5	105	136	0.77	50.4	49.6	130	80	No	Yes	Yes	No	Yes
Mean			35.7	1.5	85.9	37.3	114.0	117.7	1.0	46.2	53.8	124.0	78.0					
St. Dev			8.8	0.1	13.4	6.8	15.3	11.6	0.1	8.3	8.3	5.2	4.2					
1OB	–	M	35.1	1.75	144.0	47.0	124	142	0.87	40.1	59.9	130	80	No		–		
2OB	–	M	28.6	1.82	149.2	45.0	141	138	1.02	42.0	58.0	160	90	Yes (2)		–		
3OB	–	M	37.25	1.77	124.6	39.8	124	133	0.93	37.8	62.2	120	80	Yes (1)		–		
4OB	–	M	31.5	1.68	101.3	36.0	105	114	0.92	35.8	64.2	140	70	No				
5OB	–	F	29.0	1.70	110.2	38.1	114	130	0.88	48.3	51.7	120	80	Yes (1)		Yes[b]		
6OB	–	F	35.9	1.57	128.4	52.1	126	138	0.91	58.3	41.7	120	70	No		No[b]		
7OB	–	F	38.2	1.64	122.6	45.6	127	132	0.96	56.3	43.7	110	80	Yes (1)		Yes[b]		
8OB	–	F	44.0	1.65	88.1	32.5	109	113	0.96	42.5	57.5	110	80	No		No[b]		
9OB	–	F	39.7	1.64	124.4	46.3	123	137	0.90	42.9	57.1	120	70	No		No[b]		
10OB	–	F	24.0	1.60	103.2	40.3	104	125	0.83	48.9	51.1	120	80	Yes (1)		Yes[b]		
Mean			34.3	1.7	119.6	42.3	119.7	130.2	0.9	45.3	54.7	125.0	78.0					
St. Dev			6.0	0.1	19.1	5.9	11.6	10.0	0.1	7.5	7.5	15.1	6.3					

[a]Determined by bioelectrical impedance analysis (Human-IM Scan; DS-Medigroup, Milan, Italy). [b]Sex steroid therapy for contraceptive purpose.

Serum collection

After an overnight fasting, blood samples for each patient were collected at 7.30 h by standard venipuncture in BD Vacutainer serum separating tubes (Becton Dickinson, Plymouth, UK) and were left for 1 h at room temperature. Afterwards, blood samples were subjected to a double centrifugation: the first step at 1900×g at 4°C for 10 min, the second one at 16000×g at 4°C for 10 min. Supernatants were transferred in new tubes and subsequently frozen at −80°C for long-term storage.

Assessment of haemolysis

Serum haemoglobin was assessed with Beckman Coulter DU730 spectrophotometer using the Harboe Direct spectrophotometric method (17) with Allen correction (18): Hb (g/L) = (167.2 × A_{415} − 83.6 × A_{380} − 83.6 × A_{450}) × 1/1000 × 1/dilution in dH_2O. The considered cut-off for serum was 0.020 g/L.

RNA extraction

Small RNAs were isolated from 300 μL of serum using the miRCURY RNA Isolation Kits – Biofluids (Exiqon, Vedbaek, Denmark). miRNAs were quantified in a Qubit 2.0 Fluorometer (Thermo Fischer Scientific) using the Qubit miRNA Assay Kit (Thermo Fischer Scientific) following the manufacturer instructions.

Microarray profiling

Serum of PWS patients and obese controls, adequately matched for age, sex and BMI, was extracted and independently profiled on the Affymetrix miRNA arrays containing 2578 Human mature miRNA probe sets. RNA was labelled with the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Thermo Fischer Scientific) and hybridised on Genechip miRNA 4.0 (Thermo Fischer Scientific). The array cartridges were processed on an Affymetrix Fluidic Station 450 and scanned on an affymetrix GeneChip 3000 7G. The robust Multichip Analysis (RMA) algorithm was used to derive CEL file probe-level hybridization intensities at the gene expression levels.

In silico data analysis

miRNA Target prediction analysis was performed by using (DIANA)-micro T-CDS (v.5.0) prediction algorithm (19). The miRNA target pathway enrichment analysis was subsequently conducted with DIANA-miRPath (v.3.0) (20) considering either the predicted or the validated miRNA target repository in mirTarbase (21). The micro T-CDS threshold score for predicted targets was set at 0.8 and the P value threshold at $P<0.05$. The enrichment analysis was performed using the Fisher's exact test with a Hypergeometric distribution corrected for multiple testing by using the false discovery rate (FDR) correction.

Data analysis and statistical methods

One-way ANOVA test was used to determine gene expression differences among groups. Multiple testing correction was performed with the Benjamini–Hochberg method and FDR, corrected P values were calculated. The miRNA expression relationship among the study subgroups was analysed using Pearson correlation. Correlation matrices were generated by using Hmisc (version 4.1-1), PerformanceAnalytics (version 1.4.3541) and Corrplot (version 0.84) packages in R environment (RStudio Integrated Development for R. RStudio, Inc., Boston, MA, USA (http://www.rstudio.com/)).

Results

Serum miRNAs in PWS and non-syndromic obese subjects

We analysed the circulating miRNome of PWS vs non-syndromic obese subjects (OB) throughout the Affymetrix Genechip miRNA 4.0 array build on miRBase release 20 (22). miRNA species were classified into five quintiles based on the 'Present'/'Absent' call of the Affymetrix algorithm (23). Undetectable miRNAs or miRNAs expressed in less than five subjects were grouped together into the 1st quintile, containing 2216 out of the 2578 human mature miRNAs. The remaining quintiles ranked the miRNAs species from the rarest to the widely expressed ones.

The 2nd quintile group, including 138 miRNA species, consists of rarely detected miRNAs in both populations. Among them, miR-374a-5p, miR-4701-5p and miR-1298 were detectable only in the OB population, while miR-4800-5p and miR-7111-5p were present only in PWS. Mir-7111-5p was expressed in PWS with steatosis. Mir-382-5p, miR-345-5p, miR-532-5p, miR-4323-5p, mir-6796-3p and miR6836-3p were detectable mainly in the OB population, while the three miRNAs, miR-6721-5p, miR-2392 and miR-4486, in the PWS. The 2nd quintile miRNAs showed low average log2 signal,

very close to the background noise, making those miRNAs preferable targets for more sensitive validation techniques to further confirm microarray results (Supplementary Table 1, see section on supplementary data given at the end of this article). The 3rd and 4th quintiles consisted respectively of 54 and 45 miRNAs (Supplementary Tables 2 and 3), group most of the regulators of lipid metabolism, adipogenesis and insulin resistance pathways. Among them, miR-23a, miR-26a, miR-150 and miR-223 were reported to be involved in adipogenesis (24), insulin resistance (25, 26) and macrophage activation (27), while miR-130,miR-106b and miR-19b participate in cholesterol metabolism and efflux (14). The 5th quintile consisted of 125 miRNAs, including the 10% top expressed circulating miRNome with a log2 fold change (FC) greater than 5 (Supplementary Table 4). Among the highly expressed miRNA in serum, miR-92a-3p, mir-122, miR-185-5p, 191-5p were implicated in lipid metabolism and their association with obesity had already been proven (28). In particular, mir-92a-3p negatively correlated with the brown adipose tissue activity (29), while, the liver-enriched miR-122 was reported to work as central regulator of cholesterol and lipid metabolism (30) being upregulated in serum of obese young adults with insulin resistance (31) and subjects with non-alcoholic fatty liver disease (NAFLD) (32).

In the PWS and Angelman syndrome imprinted domain in chromosome 15q11-13, three miRNAs genes (miR-4509, miR-4715, miR-4508) were located. miR-4509 and miR-4715 were undetectable in both PWS and OB, while miR-4508 was expressed in both PWS and OB with no statistically significant differences, suggesting an alternative imprinted pattern or other coding sites within the genome.

Differentially expressed miRNAs

Thirty-four miRNAs were differently expressed between PWS and OB subjects (ANOVA *P* value <0.05, FC cut-off ±1.5) (Table 2). Thirteen miRNAs were upregulated in the serum of OB vs PWS. miR-4689, miR-151a-5p and miR-106a-5p were considered as absent in the PWS population from the A/P affymetrix algorithm, having an average signal near to the background. Three miRNAs (miR-24-3p, miR-122, miR-23a-3p) had a FC >10. Twenty-one miRNAs were upregulated in the serum of PWS subjects vs OB, however, with a FC <4. Only few differences existed between sexes, involving miR-4706, miR-193a-5p, miR-146a-5p, miR-425-5p, miR-191 and

miR23a-3p (Table 3). Interestingly miR-23a-3p had a low average log2 signal in PWS females.

When considering the presence of liver steatosis in the non-syndromic obese population, a signature of 4 miRNAs differently identified the two subgroups (OB with steatosis vs OB without steatosis): miR-7107-5p was prevalently expressed in OB with steatosis; on the contrary, miR-151a-5p, miR-3620-5p and miR-4433-3p were higher in the group without steatosis. It worth to be noticed that the FC between the two categories is generally low, being higher than two only for miR-151a-5p and miR-3620-5p (FC=2.39 *P*=0.022, FC=2.18 *P*=0.009, respectively) (Table 4). A different signature of miRNAs significantly distinguished PWS with steatosis from PWS without steatosis, involving miR-619-5p, miR-4507 (FC=2.17 *P*=0.018, FC=1.66 *P*=0.029, respectively) which were higher in PWS without steatosis, and miR-4656, miR7847-3p and miR-6782-5p which were detectable only in the population without steatosis (Table 5).

The comparison between PWS and OB showed a large difference within the group with steatosis (Table 6). Five miRNAs were significantly higher in OB subjects. Among them mir-23a-3p showed the highest differential expression with a signal 35.67 times higher in OB (*P*=0.028). Conversely, miR-1273g-3p was more abundant in PWS serum, being 4.09 time higher as compared to OB (*P*=0.02). Other six miRNAs were moderately higher in PWS. Among them, miR-8075 showed a very high log2 signal in both groups (6.34±0.49; 7.46±0.73 in OB and PWS respectively).

A distinct miRNA signature of significantly different circulating miRNAs characterised the absence of liver steatosis. Twenty-six miRNAs were altered between OB and PWS, the great portion of miRNA results upregulated in the serum of PWS while only four miRNAs were upregulated in OB, such as miR-6068, miR-1281, miR-4530 and miR-122-5p (Table 7). The liver-specific miR-122-5p showed a strong upregulation in OB, being 50 times higher as compared to PWS (*P*=0.01). Indeed, the log2 average expression was 8.12±0.1.94 in the OB population compared to 2.47±2.13 of the PWS (Table 7). Considering the miRNAs in PWS subjects, miR-3178, miR-7110-5p and miR-6891-5p, miR-1273g-3p had the highest FC compared to OB (FC=5.27 *P*=0.02, FC=4.82 *P*=0.031, FC=3.87 *P*=0.007, FC=3.32 *P*=0.03, respectively). Considering the absolute expression in serum miR-3178, miR-7110-5p and miR-1273g-3p had the highest expression in PWS patients, while, miR-122-5p in OB. miR-4497, miR-7107-5p, miR-8075 and miR-5787 had a high serum expression

Table 2 Differently expressed circulating miRNAs in obese (OB) vs Prader–Willi syndrome (PWS) subjects.

Transcript ID	OB avg (log2) signal	PW avg (log2) signal	OB s.d.	PW s.d.	OB expr.	PW expr.	Fold change	*P*-value
hsa-miR-3178	4.92	6.75	0.91	1.89	T	T	−3.55	0.0408
hsa-miR-1273g-3p	2.72	4.41	0.91	1.09	T	T	−3.22	0.0030
hsa-miR-6816-5p	2.53	3.94	0.61	1.29	T	T	−2.66	0.0349
hsa-miR-6805-5p	2.08	3.33	0.96	1.14	T	T	−2.38	0.0176
hsa-miR-4497	5.86	7.01	1.09	0.7	T	T	−2.22	0.0064
hsa-miR-619-5p	2.34	3.49	0.48	1.09	T	T	−2.21	0.0048
hsa-miR-320e	1.66	2.71	0.73	0.93	T	T	−2.07	0.0100
hsa-miR-7150	1.6	2.65	0.8	0.81	T	T	−2.07	0.0216
hsa-miR-4706	2.06	3.1	0.45	1.1	T	T	−2.05	0.0005
hsa-miR-4429	2.97	3.97	0.84	0.98	T	T	−1.99	0.0075
hsa-miR-6891-5p	1.85	2.84	0.92	0.73	T	T	−1.99	0.0302
hsa-miR-8075	5.88	6.8	0.58	0.59	T	T	−1.9	0.0010
hsa-miR-1268a	1.33	2.24	0.46	0.95	T	T	−1.87	0.0113
hsa-miR-6749-5p	2.1	2.9	0.53	0.59	T	T	−1.74	0.0099
hsa-miR-6722-3p	1.88	2.67	0.61	0.93	T	T	−1.73	0.0216
hsa-miR-6085	2.65	3.44	0.56	0.83	T	T	−1.72	0.0027
hsa-miR-6794-5p	2.83	3.59	0.55	0.72	T	T	−1.68	0.0217
hsa-miR-5787	6.05	6.79	0.31	0.46	T	T	−1.67	4.19E-06
hsa-miR-1275	1.54	2.28	0.51	0.51	T	T	−1.67	0.0066
hsa-miR-6779-5p	1.34	2.04	0.52	0.6	T	T	−1.62	0.0192
hsa-miR-4632-5p	1.3	1.93	0.5	0.57	F	T	−1.54	0.0231
hsa-miR-4689	2.1	1.29	0.74	0.64	T	F	1.76	0.0271
hsa-miR-1281	3.27	2.26	0.62	0.85	T	T	2.01	0.0027
hsa-miR-151a-5p	2.44	1.04	1.41	1.01	T	F	2.63	0.0104
hsa-miR-4530	3.37	1.74	0.98	0.64	T	T	3.11	0.0022
hsa-miR-106a-5p	3.75	1.18	1.96	1.76	T	F	5.95	0.0330
hsa-miR-191-5p	6.08	3.48	2.09	1.81	T	T	6.1	0.0435
hsa-miR-425-5p	3.83	1.21	1.47	1.62	T	T	6.14	0.0301
hsa-miR-106b-5p	3.89	1.21	1.74	1.45	T	T	6.38	0.0136
hsa-miR-92a-3p	6.65	3.97	2.39	2.11	T	T	6.42	0.0486
hsa-miR-93-5p	4.53	1.47	2.37	1.95	T	T	8.35	0.0361
hsa-miR-24-3p	5.09	1.35	3	2.12	T	T	13.32	0.0238
hsa-miR-122-5p	6.92	2.84	2.39	1.97	T	T	17	0.0078
hsa-miR-23a-3p	6.16	2.05	2.59	2.33	T	T	17.25	0.0239

in both populations (Table 7). miR-151a-5p resulted expressed in OB only, with a log2 signal of 2.95 ± 1.52.

In silico circulating miRNA target prediction and pathways enrichment analysis

The differently upregulated miRNA between OB and PWS were further considered for an *in silico* miRNA target identification and GO enrichment analysis.

To identify the significantly enriched GO pathways a miRPath v 3.0 (20) (DIANA tool) analysis was performed.

The experimentally validated miRNA targets of the upregulated miRNA signature in OB were searched in mirTarBase v.7.0 integrated in DIANA miRPath v 3.0. Among the 13 selected miRNAs, only two (miR-4530 and miR-4689) had no experimentally validated target identified yet (Supplementary File 1). Initially the enrichment analysis for each miRNA was performed and a *P* value for every pathway was calculated. Merged *P* values were then calculated combining the previously significance levels using Fisher's meta-analysis method, resulting in the probability that a determinate pathway

Table 3 Differently expressed circulating miRNAs between sexes in the two considered populations.

Transcript ID	F avg (log2) signal	M avg (log2) signal	F s.d.	M s.d.	F expr.	M expr.	Fold change	*P*-value
hsa-miR-23a-3p	1.62	5.69	2.83	1.95	T	T	−16.82	0.0237
hsa-miR-191-5p	3.46	5.61	2.24	1.1	T	T	−4.42	0.0109
hsa-miR-425-5p	2.04	3.94	1.68	1.46	T	T	−3.73	0.0314
hsa-miR-146a-5p	1.95	3.28	1.13	1.35	T	T	−2.51	0.0448
hsa-miR-193a-5p	2.66	3.51	0.98	0.8	T	T	−1.8	0.0340
hsa-miR-4706	2.56	1.97	1.22	0.56	T	T	1.51	0.0273

Table 4 Differently expressed circulating miRNAs in obese with steatosis (OBS−) vs Obese without steatosis (OBS+).

Transcript ID	OBS+ avg (log2) signal	OBS− avg (log2) signal	OBS+ s.d.	OBS− s.d.	OBS+ expr.	OBS− expr.	Fold change	*P*-value
hsa-miR-151a-5p	1.69	2.95	1.28	1.52	T	T	−2.39	0.0225
hsa-miR-23a-5p	0.85	2	0.36	0.68	F	T	−2.21	0.0100
hsa-miR-3620-5p	2.66	3.79	0.81	0.28	T	T	−2.18	0.0093
hsa-miR-576-5p	0.36	0.97	0.41	0.15	F	F	−1.52	0.0254
hsa-miR-4433-3p	1.44	2.03	0.37	0.56	T	T	−1.5	0.0252
hsa-miR-4258	1.8	1.2	0.32	0.36	T	F	1.52	0.0011
hsa-miR-6793-3p	2	1.37	0.58	0.46	T	F	1.55	0.0212
hsa-miR-6750-3p	1.42	0.78	0.35	0.13	F	F	1.56	0.0440
hsa-miR-6880-3p	1.52	0.82	0.47	0.32	T	F	1.63	0.0163
hsa-miR-7107-5p	6.46	5.59	1.04	1.7	T	T	1.83	0.0268

is targeted by at least one miRNA out of the circulating signature in OB. We generated a Heatmap with the first 45 enriched GO biological processed pathways. Most of the highly enriched pathways consisted of general cellular metabolic processes (Fig. 1).

miR-24-3p, miR-92a-3p and miR-93-5p were highly associated to 'cellular lipid metabolic process' pathway. In addition, mir-93-5p targets were also enriched in the 'FGF receptor-signalling' pathway and 'insulin receptor-signalling' pathway (Fig. 1).

Since among the 21 miRNAs upregulated in PWS subjects, only five miRNAs (miR-320e, miR-1275, miR-1263g-3p, miR-4429 and miR-4632-5p) had experimentally validated targets (Supplementary File 2), we performed the miRPath analysis by using the identified by using the DIANA-microT-CDS tool (19) that combines miR-target predictions based on both CDS and the 3′-untranslated regions (3′-UTRs) of the RNA target (Supplementary File 2). Despite the prediction, the enrichment was performed unsatisfactory with only ten pathways involved (Fig. 2). Only few miRNAs had a significant role in the predicted pathways related to general biological processes. However, it is noteworthy the enrichment of the 'neurotrophin TRK receptor signalling' pathway since the brain-neurotrophic factor (BDNF) haploinsufficiency is associated to PWS.

Discussion

PWS is the most common genetic-derived obesity disorder, determined by the absent expression of paternal alleles in the PWS critical region on chromosome 15. Within the imprinted region, 14 known genes are located, generating protein-coding transcripts, long non-coding RNAs (lncRNAs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAS (piRNAs) and miRNAs (33). The well-defined genetic determinants, leading to this obesity-related disorder, could contribute in elucidating the molecular pathways involved in other obesity-related diseases as well. As fundamental post-transcriptional gene regulators, miRNAs have now a well-established role in cell molecular pathways, including regulatory gene networks related to obesity (34, 35). Recently, Thomou and coll. (36) have identified the adipose tissue as an important source of circulating exosomal miRNAs, working as endocrine regulators of gene expression in distant tissues. In their experiments, the adipose tissue-derived exosomal miRNAs were able to influence the expression of liver FGF21 in a mouse model. Thus, circulating miRNAs can be considered not only as potential biomarkers of the disease but, also, as an informative tool mirroring the cell mechanisms and pathways involved in obesity-related diseases.

Table 5 Differently expressed circulating miRNAs between Prader–Willi syndrome subjects with (PWS+) and without steatosis (PWS−).

Transcript ID (array design)	PWS+ avg (log2) signal	PWS− avg (log2) signal	PWS+ s.d.	PWS− s.d.	PWS+ expr.	PWS− expr.	Fold change	*P*-value
hsa-miR-619-5p	2.61	3.73	1.29	0.49	T	T	−2.17	0.0189
hsa-miR-4656	1.01	1.85	0.29	0.23	F	T	−1.79	0.0001
hsa-miR-7847-3p	0.9	1.7	0.17	0.5	F	T	−1.74	0.0005
hsa-miR-4507	1.82	2.55	0.48	0.71	T	T	−1.66	0.0294
hsa-miR-6782-5p	1.36	2	0.46	0.8	F	T	−1.56	0.0145
hsa-miR-6729-3p	1.54	0.86	0.36	0.2	F	F	1.61	0.0044

Table 6 Differently expressed circulating miRNAs between Prader–Willi syndrome subjects (PWS+) and obese with steatosis (OBS+).

Transcript ID	OBS+ avg (log2) signal	PWS+ avg (log2) signal	OBS+ s.d.	PWS+ s.d.	OBS+ expr	PWS+ expr	Fold change	*P*-value
hsa-miR-1273g-3p	2.26	4.29	0.76	1.35	T	T	−4.09	0.0208
hsa-miR-8075	6.34	7.46	0.49	0.73	T	T	−2.18	0.0173
hsa-miR-4429	2.59	3.69	0.82	1.02	T	T	−2.15	0.0381
hsa-miR-1275	1.4	2.42	0.53	0.52	T	T	−2.03	0.0361
hsa-miR-6805-5p	1.68	2.67	0.89	1.5	T	T	−1.99	0.0325
hsa-miR-6749-5p	2	2.96	0.47	0.8	T	T	−1.94	0.0278
hsa-miR-4706	2.04	2.88	0.45	0.74	T	T	−1.79	0.0298
hsa-miR-4530	3.39	1.57	1.16	0.73	T	T	3.52	0.0382
hsa-miR-425-5p	3.92	1.09	1.71	1.15	T	T	7.09	0.0371
hsa-miR-106b-5p	4.28	1.27	1.68	0.45	T	T	8.03	0.0202
hsa-miR-191-5p	6.31	3.08	2.42	1.3	T	T	9.38	0.0487
hsa-miR-23a-3p	6.64	1.48	2.86	1.74	T	T	35.67	0.0280

In our work, we analysed the circulating miRNome in a cohort of patients suffering from a genetic-related obesity disease, such as PWS, compared to an age-, BMI, sex-matched cohort of patients with essential obesity. To our knowledge, this is the first description of a comprehensive circulating miRNA profile comparing the two obese populations.

Only a small portion of the mature miRNA sequences in miRBase v.20 were detectable in the analysed serum samples ($n = 362$). Among the 13 up-regulated serum miRNA in OB, miR-24-3p and miR-122 had the highest expression. These miRNAs, together with mir-23a-3p, have been already extensively studied in obesity-related diseases for their role as regulators of lipid metabolism in liver [37, 38]. In line with our observations, Nunez Lopes and coll. [39] found that increased plasma circulating levels of miR-24 were associated with obesity and diabetes spectrum. Similarly, Wang and coll. [31] correlated the elevated circulating levels of miR-122 with the obesity degree.

Table 7 Differently expressed circulating miRNAs between Prader–Willi syndrome subjects (PWS−) and obese without steatosis (OBS−).

| Transcript ID	OBS− avg (log2) signal	PWS− avg (log2) signal	OBS− s.d.	PWS− s.d.	OBS− expr	PWS− expr	Fold change	*P*-value
hsa-miR-3178	4.2	6.6	1.08	0.71	T	T	−5.27	0.0254
hsa-miR-7110-5p	3.97	6.24	1.22	0.99	T	T	−4.82	0.0311
hsa-miR-6891-5p	1.36	3.31	0.79	0.77	T	T	−3.87	0.0072
hsa-miR-1273g-3p	3.3	5.03	1.04	0.79	T	T	−3.32	0.0323
hsa-miR-1268a	1.19	2.72	0.65	0.87	T	T	−2.89	0.0144
hsa-miR-4706	1.91	3.42	0.48	1.41	T	T	−2.87	0.0018
hsa-miR-4497	5.7	7.2	1.14	0.53	T	T	−2.83	0.0191
hsa-miR-619-5p	2.25	3.73	0.34	0.49	T	T	−2.79	0.0011
hsa-miR-7107-5p	5.59	6.79	1.7	0.76	T	T	−2.3	0.0477
hsa-miR-6798-5p	2.13	3.31	0.65	0.88	T	T	−2.26	0.0187
hsa-miR-8075	5.72	6.79	0.68	0.44	T	T	−2.09	0.0095
hsa-miR-7150	1.27	2.27	0.44	0.62	T	T	−2	0.0075
hsa-miR-6769b-5p	1.97	2.88	0.43	0.44	T	T	−1.88	0.0081
hsa-miR-6085	2.53	3.39	0.63	0.6	T	T	−1.81	0.0018
hsa-miR-5787	5.94	6.76	0.37	0.64	T	T	−1.77	0.0001
hsa-miR-6722-3p	2.1	2.91	0.84	0.84	T	T	−1.75	0.0498
hsa-miR-320e	1.93	2.73	0.96	1	T	T	−1.74	0.0408
hsa-miR-4507	1.85	2.55	0.67	0.71	T	T	−1.63	0.0393
hsa-mir-550a-1	1.92	2.57	0.3	0.42	T	T	−1.58	0.0321
hsa-mir-550a-2	1.92	2.57	0.3	0.42	T	T	−1.58	0.0321
hsa-mir-550a-3	1.92	2.57	0.3	0.42	T	T	−1.58	0.0321
hsa-miR-6068	3.59	2.78	0.93	1.01	T	T	1.76	0.0203
hsa-miR-1281	3.18	1.8	0.77	0.69	T	T	2.59	0.0061
hsa-miR-4530	3.33	1.79	0.84	0.62	T	T	2.9	0.0112
hsa-miR-151a-5p	2.95	0.68	1.52	1.41	T	F	4.84	0.0200
hsa-miR-122-5p	8.12	2.47	1.94	2.13	T	T	50.02	0.0106
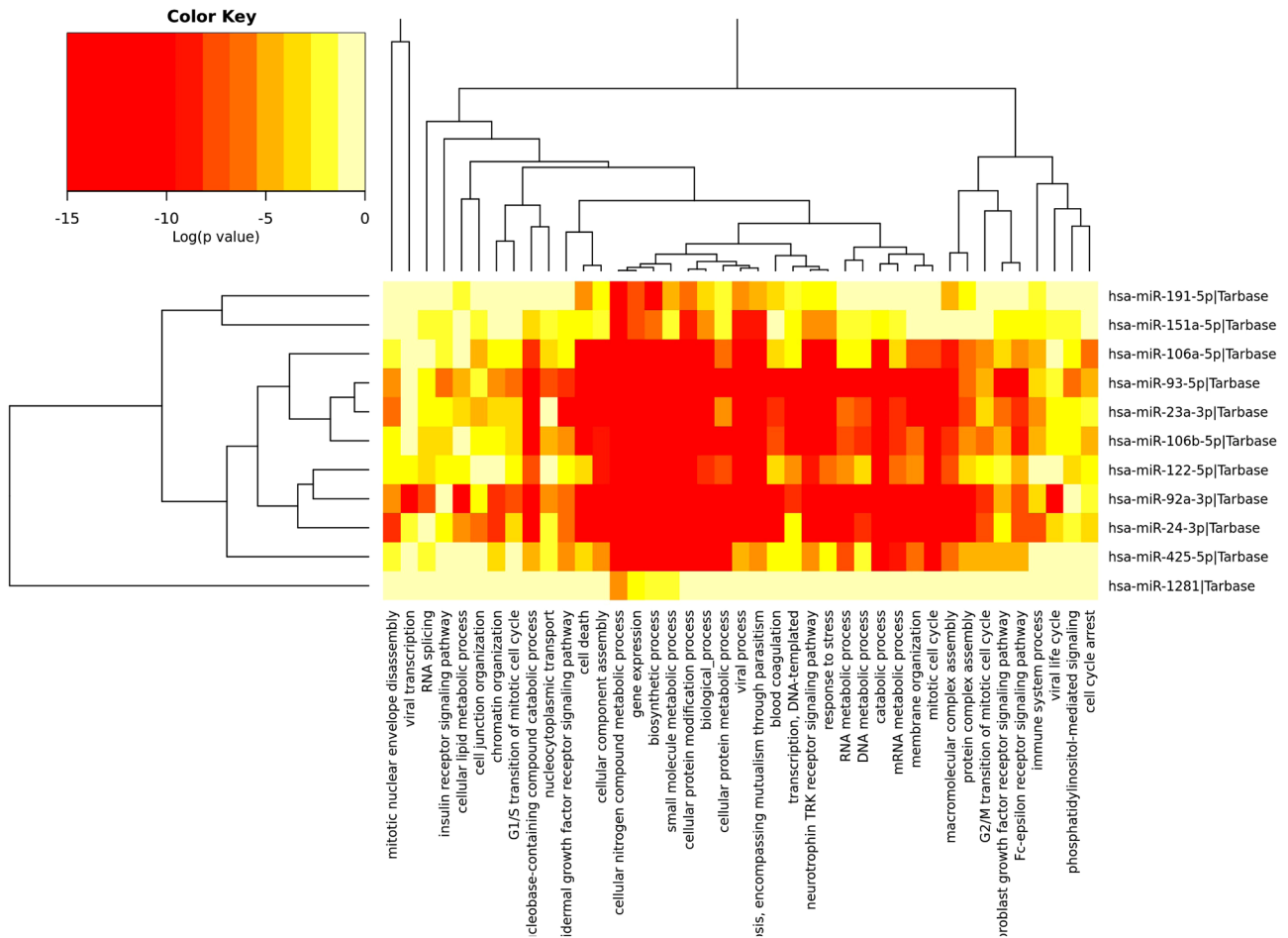
Figure 1
Heatmap of the top 45 enriched pathways related to the validated mRNA targets of the up-regulated miRNA in OB. The colour scale encodes the amount of *P*-value in logarithmic scale. The enrichment analysis was performed by using the Fisher's exact test with a hypergeometric distribution corrected for multiple testing by using the False Discovery Rate (FDR) correction.

Interestingly, the OB upregulated miR-93-5p, miR-106a-5p, miR-106b-5p and the nearly significant miR-17-5p (*P*<0.058) belong to the miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d family. Their expression is 6 to 8 folds higher in OB with a high degree of correlation among each other (Supplementary Fig. 1). In addition, they cluster together in the ontological analysis (Fig. 1), enforcing their synergism in the pathway regulation in OB. Conversely, the low expression and a poor Pearson correlation in PWS, suggested a different role of those miRNAs in this disease (Supplementary Fig. 1). miR-17-5p and miR-106a have been identified as important regulators of adipocyte differentiation, negatively regulating *BMP2*, a modulator in *MSX2/TAZ* and *PPARγ* pathway involved in the switch between adipocyte and osteoblast differentiation (40). In addition miR-106b and miR-93 are reported to be negative

regulators of brown adipocyte differentiation in mice (41). The suppression of miR-93 is associated with an increase of white adipose tissue related to an increase of adipocyte precursors through the pluripotency-associated transcription factor Tbx3 and to an increase of the adipogenesis through Sirt7 (42). Moreover, the absence of miR-106b and miR-93 lead to an upregulation of Fabp4, *PPARγ* and adiponectin (41), thus suggesting a possible role of this miRNA family as general regulators of adipogenesis. The increased adiponectin levels observed by Wu and colleagues (41) after mir-106b-93 knockdown in mice, and the low expression detected in our PWS cohort, are in line with the higher adiponectin levels measured in PWS compared with non-syndromic obese in previous studies (43). Unfortunately, we were not able to correlate the adiponectin levels to mir-93 miR-106b expression in our patients. The low expression levels of miR-17-5p,
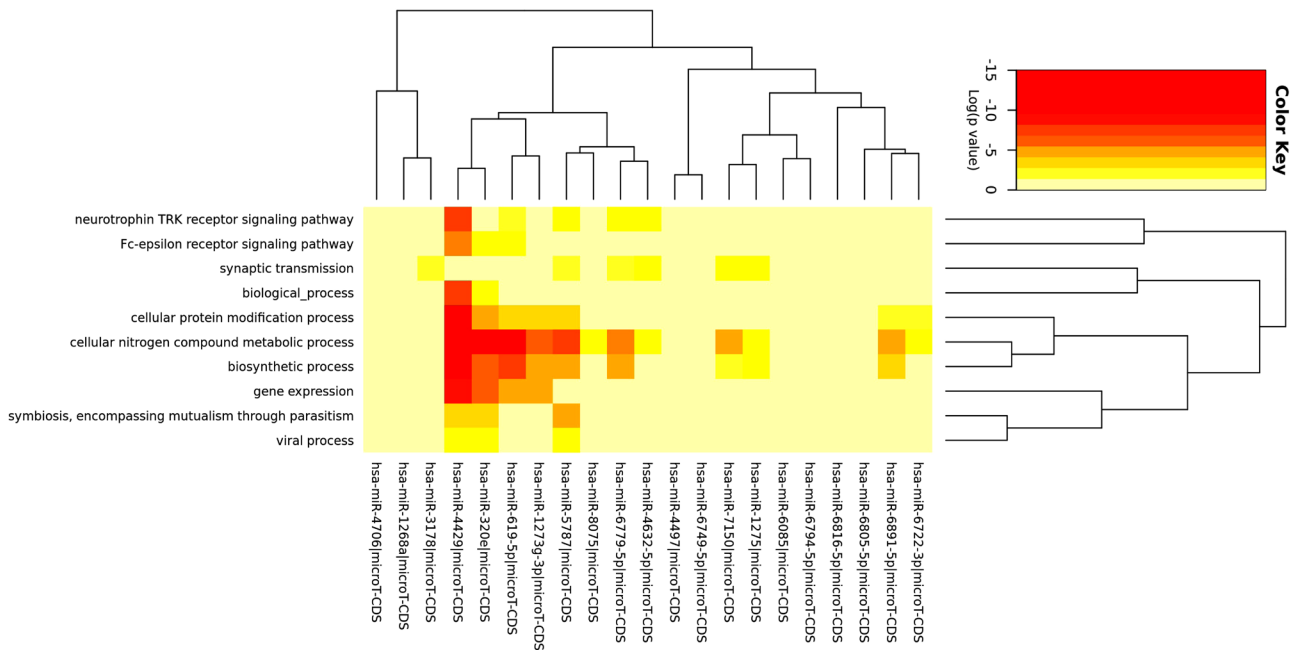
Figure 2
Heatmap of the enriched pathways related to the predicted miRNA targets of the up-regulated miRNA in PWS. The colour scale encodes the amount of *P*-value in logarithmic scale. The enrichment analysis was performed by using the Fisher's exact test with a Hypergeometric distribution corrected for multiple testing by using the False Discovery Rate (FDR) correction.

miR-106a-5p, miR-106b-5p and miR-93-5p in PWS could be related to important differences in adipogenesis and/or lipid metabolism and storage. This is also evident when comparing the PWS vs OB without steatosis in which the circulating levels of miR-122 were dramatically higher in OB compared to PWS (FC=50.02 $P<0.01$). Mir-122 is a fundamental regulator of hepatic cholesterol and fatty-acid metabolism (30), its downregulation alters the lipid metabolism in the liver (44, 45). Our observation, again, evidenced the different metabolic behaviour of the PWS compared to OB, especially in consideration that serum miR-122 have been already associated to an altered lipid profile (46). Although a distinctive circulating miRNA signature characterizes PWS subject, unfortunately only few information exists about the function of the upregulated miRNA in this syndrome. The observed differences involve some miRNA regulating adipogenesis. Mir-4429 is actively released by the adipose tissue, working as inhibitor of ACVR2B in the TGFB signalling pathways, resulting in a pro-adipogenic condition in recipient cells (47). Mir-1275 usually has a lower expression in obese subjects, compared to normal people (48), it is responsible of pre-adipocyte differentiation by targeting the transcription factor ELK1 in VAT of overweight and obese subjects (48). Since the role of the adipose tissue in the active release of exosomal miRNAs, we might explain

the observed differences in the circulating expression levels of miR-1275 and miR4429 by the different VAS/SAT (V/S) ratio and activity in the two analysed populations (9). Indeed, a distinct regulation of fat metabolism could be present in PWS. Despite the severe obesity, PWS adults have a more favourable insulin profile, associated with a different fat distribution (9, 49). The treatment with growth hormone (GH) influences the VAS/SAT (V/S) ratio with a prevalence of subcutaneous fat accumulation (50). However, more translational studies are requested to clarify the association of between mir-1275, miR-4429 and the different fat distribution in a better way.

Taking together these observations, we hypothesise that the differences in miRNA profile between PWS and OB might origin from the different metabolic pathways involved in the genetic-derived obesity vs essential obesity. Indeed, differences in the metabolic profiles have already been observed (8, 51).

These differences in adipogenesis, lipid metabolism and storage, energy expenditure could derive from a differential pathway regulation between PWS and OB. Such dissimilarities are evidenced in the ontological analysis where, apart the general metabolic processes, in OB are present enriched cellular 'lipid metabolism pathways', 'FGF receptor signalling pathway', 'TGFBr signalling pathway', 'insulin receptor signalling pathways', with a

well-established role in obesity. On the contrary, none of the mentioned pathways was enriched in PWS. The poor enrichment obtained by the mir-predicted targets highlights the absence of information in terms of miRNA characterization. This is particularly important especially in consideration of the rare disease studied. Nevertheless, the interesting enrichment in the 'neurotrophin TRK receptor signalling' pathway pointed out the role of brain-neurotrophic factors, such as BDNF.

BDNF is believed to function downstream of leptin-melanocortin signalling and to play an important role in regulating energy homeostasis and hyperphagia. Decreased levels of BDNF are associated to PWS and the study of Bochukova *et al.* (52) evidenced the downregulation of neurogenesis, neurotransmitter release, synaptic function and BDNF pathways in the hypothalamic transcriptome profile of PWS compared to normal subjects. Thus, some of the circulating miRNAs in PWS could mirror those alterations in the neurological pathways regulating food intake, energy balance and brain development.

To our knowledge, this is the first study comparing the circulating profile of a rare genetic obesity syndrome to essential (non-syndromic) obesity. Revealing the alteration in the expression of circulating small non-coding RNAs, such as miRNAs, could further contribute to identification of the different key regulatory cell pathways involved in the disease. This is particularly true when considering the emerging role of circulating miRNAs as 'messengers' in a sort of endocrine cell-to-cell communication. Our findings indicate the presence of a distinctive circulating miRNA profile between PWS and OB. The difference might derive from a differential regulation of cellular pathways, such as adipogenesis, lipid metabolism and accumulation, fat distribution and neurotrophic factor regulation. The identification of specific miRNA profile in PWS may represent an additional and valuable contribute in the clinical evaluation of hepatic impairment in these patients.

Although the rarity of PWS hampers the recruitment of wider study groups useful to draw definitive conclusions, we believe that this study performed on a small group of patients opens anyway new perspectives on molecular pathogenesis of syndromic (and non-syndromic) obesity. In addition, further validation studies should be conducted to confirm our preliminary observations in larger cohorts, mandatory to clarify the potential role of the cited miRNAs as possible circulating biomarkers to be used in clinics.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/ECC-18-0329.

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