Genome-wide miRNA profiling in plasma of pregnant women with Down syndrome fetuses

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Abstract

ABSTRACT Objective: Down syndrome (DS) belongs to the most common causes of mental retardation in men, therefore new approaches allowing its rapid and effective prenatal detection are being explored. In our study we focused on diagnostic potential of plasma microRNAs. We follow up on our previous study in DS placentas, where seven miRNAs were found as being significantly elevated. Methods: A total of 70 first-trimester plasma samples of pregnant women were included to the present study (35 samples with DS fetuses; 35 with euploid fetuses). The genome-wide miRNA profiling was performed in the preliminary study using Affymetrix GeneChip™ miRNA 4.1 Array Strips. Selected miRNAs were then included to the following validation study using qPCR. Results: Based on the current pilot study results (12 miRNAs), our previous research on CVS samples (7 miRNAs) and literature (4 miRNAs), the group of 23 miRNAs were selected for validation study. Although the results of the preliminary study were promising, the following validation step using more sensitive technology RT-PCR and larger group of samples revealed no significant differences in miRNA profiles between compared groups. Conclusion: Our results suggest that testing of the first trimester plasma miRNAs is not suitable for NIPT. Different results could be theoretically achieved at later gestational ages; however, such a result probably would have limited utilization in clinical practice.

Background

Trisomy 21 (Down syndrome; DS) is the most common chromosomal disorder with an incidence of about 1:1000 to 1:1100 live births worldwide [1]. Virtually all DS patients suffer from cognitive impairment of various degree and craniofacial abnormalities. Other phenotypic characters, like cardiovascular defects, childhood leukemia, gastrointestinal anomalies or early onset Alzheimer's disease, occur with various frequencies and exhibit interindividual heterogeneity [2]. It is generally accepted that the DS phenotype is caused by excess genetic material of chromosome 21 (Hsa21), however, specific molecular mechanisms or pathways clarifying particular DS features have still not been found [3].

Many studies focused on gene expression in various DS biological samples (fetal or placental tissues, amniotic fluid cells, fetal or maternal blood, etc.) and they have come to varied results. Some of these expression studies have even concluded that Hsa21 genes in DS are not significantly more expressed than other disomic genes [4-6].

The hypothesis that major or most serious DS manifestations are basically caused by a few genes located at relatively small region on Hsa21, so-called Down Syndrome Critical Region (DSCR), was often discussed in last years [7-9]. Nevertheless, after reanalysis of all documented cases with partial trisomy 21 (PT21), presumed DSCR was limited to highly restricted DSCR (HR-DSCR), which does not contain any known gene, except for one novel miRNA gene [10].

Thus, all observations suggest that DS phenotype is a consequence of global gene expression misregulation, which occurs mainly due to imbalanced interactions between trisomic and disomic genes

but reflects also variability of overall individual genome [11, 12]. Greater impact is apparently exerted by haploinsufficient genes, which show recognizable phenotype after one allele loss [13]. These genes, for example dual specificity tyrosine phosphorylation regulated kinase 1A (*DYRK1A)*, are also sensitive to three copies [14, 15]. Moreover, individual genomic and epigenomic backgrounds including microRNA (miRNA) gene expression regulation probably contribute to the final DS phenotype [16].

miRNAs are small (17-25 nucleotides) non-coding RNAs, which regulate gene expression at the post-transcriptional level. In most cases, binding of miRNA to target mRNA with partial complementarity induce inhibition of translation (RNA silencing). Otherwise, when high degree of complementarity between miRNA and its target is achieved, the mRNA is degraded [17]. More than 2600 human miRNAs have been described till today [18]. However, it was found that one miRNA may affect hundreds of mRNAs, as a result, miRNAs regulate virtually all cellular processes. Therefore, miRNAs were studied in relation to various pathologies, including cancer, cardiovascular diseases, diabetes or autoimmune diseases [19-22]. Extracellular miRNAs associated with pregnancy are also systematically investigated in plasma of pregnant women. Although differential miRNAs profile was described in many pregnancy-related conditions (preeclampsia, preterm delivery, ectopic pregnancy, gestational diabetes mellitus, fetal trisomies, etc.) in comparison with normal pregnancies, specific mechanisms of miRNAs release to maternal circulation are not fully elucidated [23, 24]. While most authors presume placental origin of these miRNAs as in case of cell-free DNA, some hypotheses also suggest their possible fetal origin [25, 26].

Presented study follows on our previous study comparing miRNA expression profiles in euploid and trisomic placentas, where seven miRNAs were found to be significantly up-regulated in DS placentas, three of these miRNAs are located on chromosome 21 [27]. As miRNAs are released via vesicles from placenta to maternal circulation we focused in the current study on plasma samples of pregnant women bearing DS or euploid fetuses to further investigate biological functions of miRNAs and to explore their potential for NIPT [28, 29].

Methods

The study consisted of two phases. Pilot study was performed using Affymetrix GeneChip™ miRNA 4.1 Array Strips (Affymetrix, USA) and enabled us to select broader spectrum of miRNAs with different expression between compared groups (18 samples of plasma of pregnant women included; 9 with trisomic and 9 with euploid fetuses). Based on the pilot study results, our previous research on CVS samples [27] and literature, the group of 23 miRNAs were finally selected for the subsequent validation (Table 1). TaqMan Advanced miRNA Assays (Life Technologies, USA) were used in validation study (52 samples included; 26 with trisomic and 26 with euploid fetuses).

Clinical samples

Plasma samples of pregnant women were collected between January 2015 and November 2017 at the Department of Obstetrics and Gynecology of the First Faculty of Medicine and General University Hospital in Prague, Screening Center ProfiG2 in Prague and Genvia Genetic Laboratories. All samples were obtained before chorionic villi sampling (CVS) between 11th and 14th gestational weeks from the patients with an increased fetal trisomy risk based on first trimester combined test, increased maternal age or abnormal ultrasound finding (only in case of DS pregnancies). A total of 70 samples were included in the study; 35 of them were cytogenetically confirmed to have complete fetal trisomy of chromosome 21 (47, XX, +21 or 47, XY, +21), and 35 to have normal karyotype (46, XX or 46, XY). Only non-smoking pregnant women without any medication or any subsequently identified placental pathologies (e.g., preeclamspia), which can affect overall miRNA profile, were included to our study [55, 56]. There were no statistically significant differences between compared groups of samples in maternal age, BMI, fetal sex or samples storage time (Table 3).

The study was approved by the Ethical Committee of the First Faculty of Medicine, Charles University and General University Hospital in Prague. The informed consents were obtained from all participants.

Table 3

Sample processing and miRNA isolation

Plasma separation and storage

Peripheral blood samples were collected by venipuncture using cell-free DNA BCT tubes (Streck, USA) to prevent coagulation. Tubes were stored at room temperature and processed within 6 hours after sampling at the Institute of Biology and Medical Genetics of the First Faculty of Medicine and General University Hospital in Prague and Genvia Genetic Laboratories. Two-step centrifugation was performed to obtain plasma samples from peripheral blood samples: 1) 1100g/10min at 10˚C and 2) 14500g/10min at room temperature. Plasma samples were finally frozen to -80˚C.

Optimization of the miRNA extraction

In an effort to achieve the highest miRNA yield, we tested 6 different miRNA isolation kits - NucleoSpin miRNA Plasma (Macherey-Nagel, Germany), miRCURY RNA Isolation Kit – Biofluids (Exiqon, Denmark), miRNeasy Serum/Plasma (Qiagen, Germany), mirVana PARIS (Ambion, Life Technologies, USA), QIAamp Circulating Nucleic Acid (Qiagen) and PME microRNAs Extraction Kit (Analytic Jena, Germany) - with various input (200–2000µl) and elution (14-50µl) volumes. Improvement of miRNA extraction using vacuum concentrator (SpeedVac, Thermo Fisher Scientific) and glycogen (Roche Diagnostics, USA and Invitrogen, Thermo Fisher Scientific, USA) were also tested. Concentration and quality of isolated miRNA samples were then evaluated

using three different approaches:

1) Fluorometer Qubit 3.0 with microRNA Assay Kit (Thermo Fisher Scientific)

2) Spectrophotometer (IMPLEN, Germany)

3) Quantitative reverse transcription PCR (RT-qPCR) with TaqMan MicroRNA Reverse Transcription Kit and selected TaqMan microRNA assays using QuantStudio 12K Flex (Applied Biosystems)

Four best variants of sample processing were compared directly on the array strip prior to the pilot study. The best combination was finally selected for the clinical samples' preparation. Detailed optimization procedure is provided in Supplement information (S1).

miRNA isolation

Total RNA enriched for small RNAs was extracted from 900µl of plasma using NucleoSpin miRNA Plasma kit and eluted with 20µl of supplied elution buffer. The whole procedure was performed to achieve the highest yield based on previous optimization and in accordance with manufacturer's recommendations. No enhancers like RNA carrier or glycogen were added. Before proceeding to the microarray (pilot study) or reverse transcription (validation study) step, miRNA concentration of all samples was measured using fluorometer (Qubit 3.0), total RNA concentration was determined by spectrophotometer (IMPLEN). While miRNA concentration of all samples ranged between 2-3ng/µl, concentrations of total RNA as measured with spectrophotometer were about ten times higher (20-30ng/µl).

Pilot study - genome-wide miRNA profiling

Total RNA (130ng) enriched for low molecular weight RNA from each sample was labelled using FlashTag Biotin HSR RNA Labelling Kit (Affymetrix) on GeneAtlas Hybridization Station (Affymetrix) and subsequently it was processed using GeneAtlas Hybridization, Wash and Stain Kit for miRNA Array Strips (Applied Biosystems) on GeneAtlas Fluidics Station (Affymetrix) according to the manufacturer's instructions. Array strip fluorescence intensities were finally determined using GeneAtlas Imaging Station (Affymetrix). Raw data were processed and visualized using Partek Genomics Suite software (Partek, USA).

Selection of the miRNA set for the validation study

A total of 23 miRNAs were selected for the validation study (Table 1). The set of miRNAs consisted of three groups:

1) seven miRNAs verified as being overexpressed in DS placentas in our previous study [27]

2) 12 miRNAs with significantly different expression levels between euploid and DS plasma samples of pregnant women according to the results of the pilot study (p-value ≤ 0.05; fold change ≥ 1.5)

3) four miRNAs which did not fulfil the above conditions but were reported in the literature as being possibly associated with Down syndrome pathophysiology [26, 30, 31].

Validation study

The same total miRNAs input (4ng) from each sample based on fluorometer (Qubit 3.0) measurement was reverse-transcribed using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems). Expression of each miRNA was consequently determined using quantitative real-time PCR (qPCR) with TaqMan Advanced miRNA Assays and TaqMan Fast Advanced Master Mix (Applied Biosystems). We have followed the procedure recommended by the manufacturer. All reactions were performed in triplicate. miRNAs' expression levels were detected on QuantStudio 12K Flex Real-time PCR System (Applied Biosystems).

Data analysis

Pilot study

Raw results of miRNA array strips were evaluated using Partek Genomics Suite software (Partek, USA). One-way ANOVA with a cut-off p-value ≤ 0.05 and fold change (FC) ≥ 1.5 was used for detection of differentially expressed miRNAs. To control the false discovery rate, the Benjamini-Hochberg correction was applied. All visualisations, such as heatmaps, were prepared using Partek software as well. Samples were normalized with the same miRNAs input volume.

Validation study

For the initial data processing, the QuantStudio 12K Flex Software v1.1.2 and ExpressionSuite software v1.0.3 (Thermo Fisher Scientific) were used. The qPCR results normalized with the same miRNAs input were consequently statistically evaluated using qBase+ v2.4 software (Biogazelle, Belgium). Euploid and DS fetus expression data were compared using a nonparametric Mann-Whitney test with corrected cut-off p-value ≤ 0.05 and fold change (FC) ≥ 2.

Results

Pilot study

Initial study phase served to identify a wider panel of potentially deregulated miRNAs in plasma of pregnant women with DS fetuses. Thus, the genome-wide analysis, which allows detect expression of all miRNAs listed in mirBase v.20 in one reaction, was selected. A total of 18 plasma samples (9 with fetal trisomy 21; 9 with normal karyotype) were loaded into Affymetrix miRNA array strips in the pilot study. Clear separation of compared groups of samples based on principal component analysis (PCA) is visualised on Figure 1.

Twelve miRNAs which most significantly discriminated the two compared groups of samples were selected using one-way ANOVA (p-value ≤ 0.05; FC ≥ 1.5; Table 1). Half of these miRNAs were up-regulated in samples with fetal trisomy, half of them were down-regulated. None of 12 selected miRNAs are on the chromosome 21. Seven miRNAs that were validated as significantly elevated in DS placentas in our previous study [27] were not among the differentially expressed miRNAs in the current pilot study with plasma samples.

Figure 1

Validation study

Finally, a total of 23 miRNAs were selected for the validation study (Table 1). Apart from the 12 miRNAs identified in the abovementioned pilot study, the selection was based on the results of our previous study focused on placenta samples (7 miRNA) and the results of current pilot study in combination with information in literature (4 miRNAs) [26, 30-33].

The expressions of 23 selected miRNAs were determined using qPCR analysis with individual miRNA assays in an independent set of samples. A total of 52 plasma samples (26 with fetal trisomy 21; 26 with normal karyotype) were included. Differences in miRNA levels between compared groups of samples were evaluated using non-parametric Mann-Whitney *U*-test (p-value ≤ 0.05; FC ≥ 2; Benjamini–Hochberg correction). None of the 23 tested miRNAs was confirmed to be significantly differentially expressed between plasma samples with fetal trisomy and samples with confirmed normal karyotype of the fetuses.

Table 1

Discussion

In our previous research we performed miRNA expression profiling of CVS samples from euploid and trisomic pregnancies [27]. A total of 80 CVS samples (40 with normal karyotype, 40 with trisomy of chromosome 21) were included. Seven miRNAs were finally verified using qPCR as significantly up-regulated in DS placentas (miR-99a, miR-542-5p, miR-10b, miR-125b, miR-

615, let-7c and miR-654), three of them were located on chromosome 21 (miR-99a, miR-125b, let-7c). Except for various essential biological processes, we identified many genes involved in placenta development (*GJA1*, *CDH11*, *EGF*, *ERVW-1*, *ERVFRD-1*, *LEP* or *INHA*) as being potentially altered by elevated miRNA levels.

It was found that human placenta expresses more than 500 different miRNAs, some of them specific for this tissue [34]. Placental-specific miRNAs are expressed from three main clusters - C14MC (chromosome 14 miRNA cluster), C19MC and miR-371-3 [35]. Typical changes in expression of miRNAs from these three clusters during pregnancy suggest their potential involvement in physiological processes [36]. For example, expression of miRNAs from cluster C19MC increases continually from first to third trimester and closely correlates with placenta growth [37]. miRNAs are released from placenta, primarily from placental trophoblast, into maternal and fetal circulation mainly via exosomes [28]. However, placenta is not the only determinant of pregnancy-associated miRNA levels in maternal and fetal blood, another source or mechanism influencing these levels is probably involved [29]. Hypothesis that miRNAs are somehow transported from fetus into the maternal circulation and vice versa is still unproven [26].

To further extend our knowledge about biological functions of miRNAs and assess their diagnostic potential, we focused in the follow-up study on maternal plasma samples. To our best knowledge, this is the first study performing genome-wide miRNA profiling in plasma samples of pregnant women with euploid and DS fetuses. All 70 plasma samples were obtained immediately before CVS sampling, so between 11^{th} and 14^{th} gestational week.

Methods analyzing genome-wide miRNA profiling (NGS or arrays) require a high miRNAs input, which is challenging in case of plasma samples. Therefore, most of the studies analyze only selected group of miRNAs in plasma using qPCR, where a much smaller input is needed or perform genome-wide analysis of whole maternal blood, where overwhelming background from maternal blood cells makes it virtually impossible to analyze cell-free nucleic acids from placenta [32].

To achieve the highest yield and purity of miRNAs from plasma for the purposes of Affymetrix miRNA array strips we performed exhaustive and systematic method optimization (Materials and Methods; S1). Utilization of miRNA arrays enabled us to evaluate all miRNAs listed in miRBase v.20 in one reaction. Twelve miRNAs were identified as being significantly dysregulated between compared groups of samples.

Nevertheless, promising results from the initial study phase were not verified in subsequent validation phase using more sensitive method RT-PCR and larger group of samples.

We could not select a single miRNA that would discriminate euploid and DS pregnancies on the plasma level. However, clear separation of compared groups is visible when comparing the levels of the larger group of most dysregulated miRNAs obtained from miRNA arrays (Figure 2).

Several articles comparing miRNA levels in plasma of pregnant women bearing euploid and DS fetuses have been published so far (Table 2). Nevertheless, these studies may have possibly come to different results due to different workflow used. The lack of a standardized normalization strategy represents a general issue in case of plasma miRNAs evaluation. Various reference miRNAs are used for

normalization of raw expression data. For example, miR-16 is often selected as a reference target, but it was found to be very susceptible to hemolysis [38]. Small nuclear or nucleolar RNAs are suitable only for normalization of samples where nuclear material is expected, but not for plasma samples [39]. On the other hand, global mean normalization is applicable only for larger miRNA set (>100 miRNAs) [40]. To prevent distortion of our results, we decided to normalize our data with the same total miRNAs input as described previously [41].

Figure 2

Kotlabova et al. performed expression analysis of five miRNAs from chromosome 21 (miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) using qPCR with normalization to reference miRNAs – miR-16 and let-7d [42]. Nevertheless, they found no differences between selected miRNA levels in compared groups of samples (12 pregnancies with DS fetuses; 12 control samples). Another study evaluating 14 miRNAs from Hsa21 (including five miRNAs which Kotlabova et al. focused on) also using qPCR with normalization to U6 snRNA was published by Erturk et al. [24]. They compared 33 euploid and 23 trisomic pregnancies and found two miRNAs - miR-99a and miR-3156, which were elevated in DS pregnancies. The most comprehensive study so far has been carried out by Kamhieh-Milz et al. [26]. A group of 1043 miRNAs were analyzed using high-throughput qPCR SmartChip Human miRNA Panel, nevertheless, a very small number of samples were included (7 DS fetuses; 7 controls). Using combination of three different normalization strategies (corrected threshold cycle values, normalized relative quantities and combination of both methods together) they found 36 miRNAs to be differentially expressed in DS versus control pregnancies, neither miR-99a nor miR-3156 were among them. The latest work on the topic was published by Zbucka-Kretowska et al. [43]. They examined levels of 800 miRNAs using NanoString technology within 12 DS pregnancies and 12 controls. Using normalization to geometric mean of top 100 probes (global mean), the group of 13 miRNAs was found to be deregulated.

Table 2

Except the study of Kotlabova, remaining three studies apparently did not apply any correction for multiple testing. Omitting this correction can lead to a false positive results, especially in the case of a high number of comparisons and small sample size, as in case of Kamhieh-Milz or Zbucka-Kretowska studies [44]. Moreover, Zbucka-Kretowska et al. themselves reported that using Benjamini-Hochberg's correction they would not reach any statistically significant results.

Our study included samples from early gestational weeks (11^{th}-14^{th}), which would allow potential utilization of miRNA markers found for early NIPT. However, our results from the validation study demonstrate, that levels of pregnancy associated miRNAs are apparently too low in such early pregnancies. Analysis of samples from later gestational weeks would potentially lead to different results, but without required potential for early diagnosis. Differences in gestational age could also contribute to the discrepancies between results of studies compared (Table 2).

Next to the different detection platforms, various preanalytical steps in sample handling, like sample storage conditions (time, temperature), type of preservative tube, concrete blood centrifugation conditions for plasma separation, plasma input volume to isolation or type of miRNA isolation (see supplement materials; S1), have also proven impact on the results achieved [38, 45, 46].

Regardless of different procedures and data processing, none of the studies comparing plasma samples from euploid and DS pregnancies found any miRNA, which could discriminate compared groups in all cases. So, it seems that miRNAs determination in plasma of pregnant women is not applicable for NIPT of fetal DS.

Since most of placental miRNAs are released to the circulation of pregnant woman via exosomes [28], it would be interesting in the future study to focus specifically on exosomal miRNAs. Exosomal miRNAs may be overlapped in the pool of total plasmatic miRNAs by other abundant miRNAs associated with RNA-binding proteins or derived from apoptotic cells. Exosomes are specific subtype of extracellular vesicles, which probably play a significant role in intercellular communication pathways involved in placentation, formation of vascular system between the mother and fetus or inducing of maternal immune tolerance to the fetus [47-50]. Therefore, exosomes could have potential as early non-invasive biomarkers of various pregnancy complications especially connected to placenta development. Indeed, exosomes are currently intensively studied in relation to preeclampsia [51, 52]. As Down syndrome pregnancies are also complicated by abnormal placentation [53], exosomes released from such an impaired placenta could be also promising markers for early detection of Down syndrome fetuses from maternal circulation. So far, miRNAs from circulating nanoparticles have only been studied in young individuals with DS and their siblings with promising results achieved [54].

Conclusions

Previously, we found dysregulated miRNA levels in DS placentas that potentially interfere with essential biological pathways. In our current study, we focused on plasma of pregnant women to explore whether overexpressed placental miRNAs are also detectable in maternal circulation and therefore applicable for NIPT. To our best knowledge, this is the first study performing genome-wide profiling of plasmatic miRNAs on such a large cohort of first trimester pregnant women with DS fetuses. However, we could not conclusively demonstrate differences in miRNA levels in the first trimester plasma of pregnant women with euploid and DS fetuses. The main reason probably was the high background of maternal miRNAs, which did not allow detection of potential differences in pregnancy-associated miRNAs at such early

pregnancies. Nevertheless, further research will be needed to clarify the role of miRNAs in DS pathophysiology.

Declarations

Ethics approval and consent to participate:

The study was approved by the Ethical Committee of the First Faculty of Medicine, Charles University and General University Hospital in Prague. The informed consents were obtained from all participants.

Consent for publication:

Not applicable.

Availability of data and material:

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

IZ: Co-designed the study, performed experiments, analysed data, co-wrote the paper.

BC: Performed experiments.

OŠ: Analysed data and co-wrote the paper.

MKo: Co-designed the study, co-wrote the paper.

EP: Performed bioinformatic analyses, co-wrote the paper.

MB: Co-designed the study, interpreted the patient data.

MKr: Co-designed the study, interpreted the patient data.

PC: Co-designed the study, interpreted the patient data, co-wrote the paper.

AH: Supervised the research, designed the experiments, co-wrote the paper.

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Tables

Table 1: Set of 23 miRNAs selected for the validation study

Selection based on		MirBase Accession No	Systematic ID	Up/Down-regulated
Validated results of previous study on CVS		MIMAT0000097	miR-99a	Up-regulated
		MIMAT0003340	miR-542-5p	Up-regulated
		MIMAT0000254	miR-10b	Up-regulated
		MIMAT0000423	miR-125b	Up-regulated
		MIMAT0003783	miR-615	Up-regulated
		MIMAT0000064	hsa-let-7c	Up-regulated
		MIMAT0003330	miR-654	Up-regulated
Current pilot study on plasma samples (Affymetrix miRNA 4.1 array strips); $p \leq 0.05$	$p \leq 0.05$ + $FC \geq 1.5$	MIMAT0017991	hsa-miR-3613-3p	Up-regulated
		MIMAT0000062	hsa-let-7a-5p	Down-regulated
		MIMAT0000065	hsa-let-7d-5p	Up-regulated
		MIMAT0019745	hsa-miR-4668-5p	Down-regulated
		MIMAT0000421	hsa-miR-122-5p	Up-regulated
		MIMAT0002871	hsa-miR-500a-3p	Up-regulated
		MIMAT0000732	hsa-miR-378a-3p	Up-regulated
		MIMAT0005929	hsa-miR-1275	Down-regulated
		MIMAT0004614	hsa-miR-193a-5p	Down-regulated
		MIMAT0025478	hsa-miR-6511a-5p	Down-regulated
		MIMAT0027682	hsa-miR-6891-5p	Down-regulated
		MIMAT0004983	hsa-miR-940	Up-regulated
	+ literature	MIMAT0005898	hsa-miR-1246	-
		MIMAT0002824	hsa-miR-498	-
		MIMAT0000101	hsa-miR-103a-3p	-
		MIMAT0000104	hsa-miR-107	-

Table 2: Overview of the articles on the topic

Study	Included miRNAs	Samples (C/T21)	GW	Normalization	Correction
Kotlabova et al.	5	12/12	18.5	miR-16; let-7d	Bonferroni
Erturk et al.	14	33/23	17-18	U6 snRNA	No
Zbucka-Kretowska et al.	800	12/12	15-18	global mean	No
Kamhieh-Milz et al.	1043	7/7	14.1	multiple strategies	No
Our study	**all in miRBase v.20**	**35/35**	**13.3**	**input volume**	**B-H**

Abbreviations: C – controls; T21 – samples with fetal trisomy of 21; GW – average gestational week; B-H – Benjamini-Hochberg

Table 3: Clinical characteristics of individuals included to the study

	Maternal age	BMI	Fetal sex		Gest. age	N
Pilot study	Average ± SD	Average ± SD	Female	Male	Average ± SD	
Euploid fetuses	34.8 ± 1.6	23.8 ± 3.1	4	5	13.3 ± 0.3	9
DS fetuses	36.9 ± 3.3	23.2 ± 2.1	4	5	13.2 ± 0.4	9
Validation study						
Euploid fetuses	33.3 ± 4.8	23.2 ± 3.8	13	13	13.4 ± 1.3	26
DS fetuses	34.4 ± 6.3	24.1 ± 4.5	10	12	13.5 ± 0.8	26

Figures

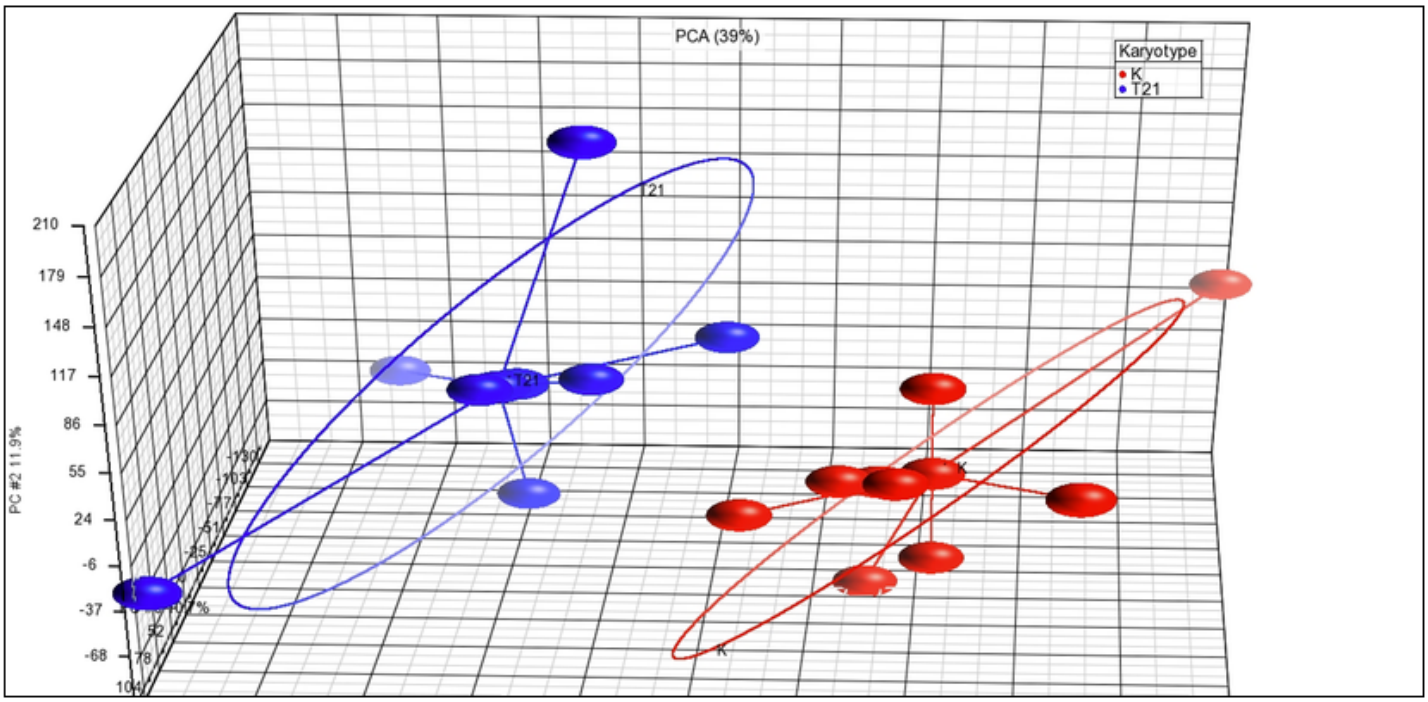

Figure 1

Scatter plot displaying compared groups of samples after principal component analysis (PCA). Samples with fetal trisomy (blue) are clearly separated from samples with euploid fetuses (red).

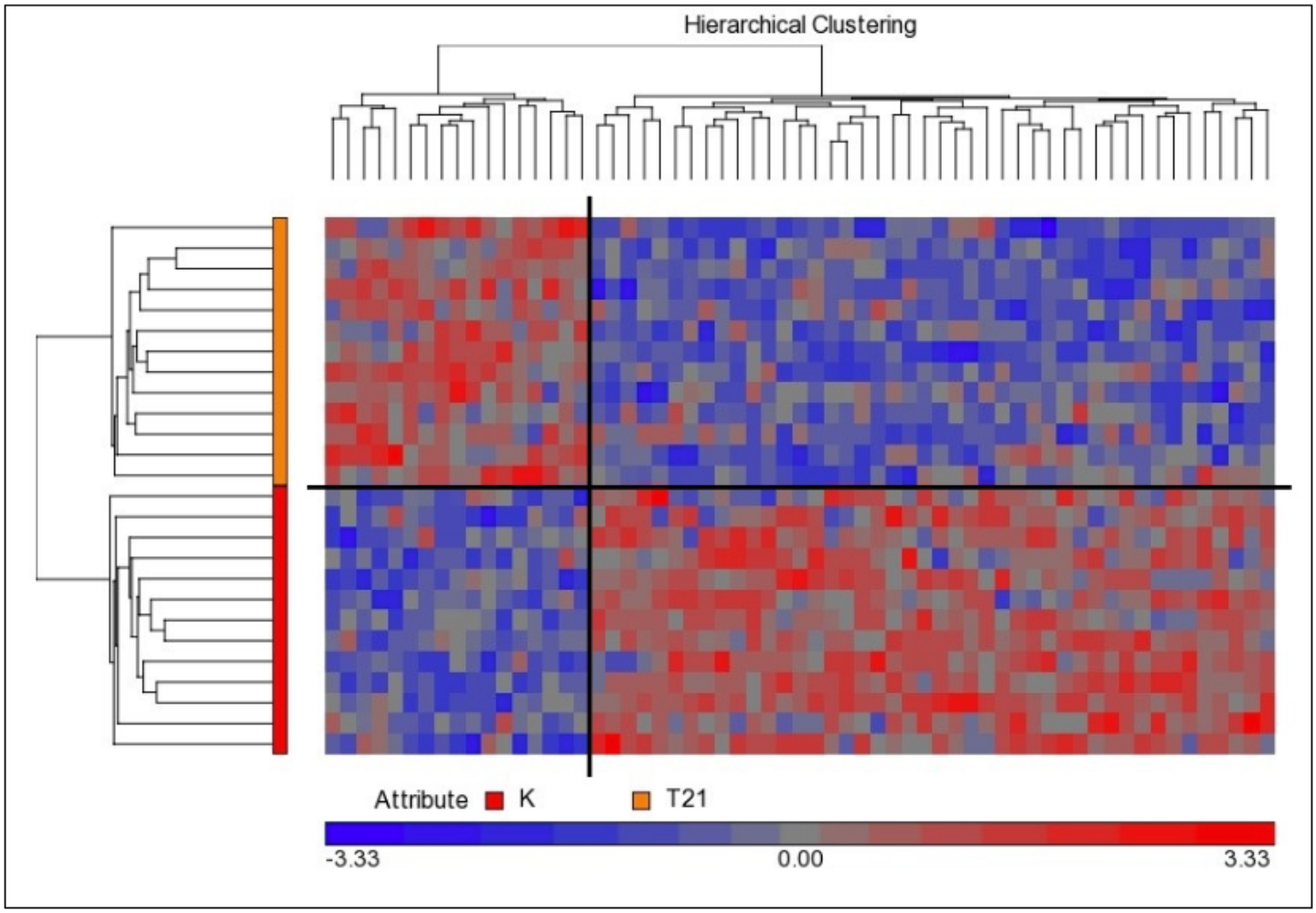

Figure 2

Heatmap displaying clear separation of pregnancies with DS fetuses (T21; orange) in comparison with controls (K; red) according to expressions of 61 miRNAs with lowest p-value (fold change value was not considered). These data are based on the results from the pilot study (miRNA arrays). Most of the deregulated miRNAs are down-regulated in DS group of plasma samples (44)

Supplementary Files

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