Variability in cerebrospinal fluid microRNAs through life

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ABSTRACT

The development of human brain starts in the first weeks of embryo differentiation. However, there are many relevant neurodevelopmental processes that take place after birth and during lifespan. Such a fine and changing scenario requires coordinated expression of thousands of genes to achieve the proper specialization and inter-connectivity. In this context, microRNAs (miRNAs), which can modulate mRNA stability and translation, are gaining recognition for their involvement in both brain development and neurodevelopmental disorders. Therefore, cerebrospinal fluid (CSF) miRNAs should be perfectly differentiated in relevant age periods. In this study, we aimed to highlight the biological variability of miRNA expression in the CSF throughout life, which is also crucial for biomarker discovery in CNS pathologies, especially in children, where they are desperately needed. **METHODS**: We analyzed the CSF microRNAome of 14 healthy children (aged 0-7.4 years) by smallRNA-Seq and compared it with previously published data in adults (N=7) and elders (N=11). **RESULTS**: miR-423-5p and miR-22-3p were overexpressed in the <1 and >3 year groups, respectively. Additionally, we detected 18 miRNAs that reached their highest peak of expression at different time-points during lifespan and sets of miRNAs that were exclusively expressed in a specific age group. On the contrary, miR-191-5p

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showed stable expression in CSF from the first year of life. **CONCLUSION**: Our results remark the complex differential miRNA expression profile that can be observed through life, which underlines the need for including appropriate age-matched controls when the expression of CSF miRNAs is analyzed in different pathological contexts.

GRAPHICAL ABSTRACT
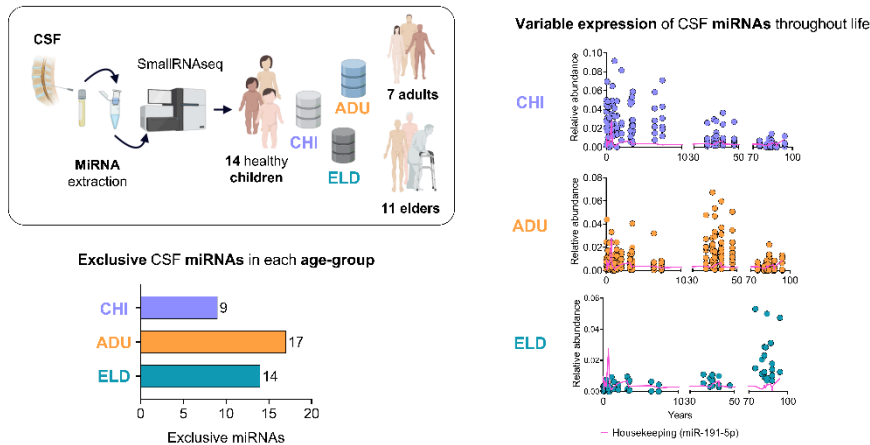
KEYWORDS: Cerebrospinal fluid; MicroRNAs; Children; Biomarkers; Biological variability

INTRODUCTION

The development of human brain starts in the first weeks of embryo differentiation. While most neurons are generated prenatally, there are many processes that are relevant for neurodevelopment that take place after birth, including proliferation and maturation of other cell types, such as oligodendrocytes, astrocytes and microglia; production of synapses followed by selective pruning; and myelination of axons [1]. In fact, the brain is known for its plasticity, which allows us to adapt to changing physiological and environmental conditions. Current data suggests that this plasticity encompasses many distinct phenomena, some of which operate across most or all of the lifespan, and others that operate exclusively in early development [1].

Such a fine and changing scenario requires coordinated expression of thousands of genes to achieve the proper specialization and inter-connectivity. In order to achieve this coordinated expression, a fine regulatory mechanism is needed. In this context, microRNAs (miRNAs) are a group of small non-coding RNAs that have an important regulatory role in gene expression at the post-transcriptional level. They can bind the 3´ untranslated region (UTR) of their target mRNAs, including those of relevance in neurodevelopment, promoting mRNA cleavage, destabilization or inhibition of translation [2]. In fact, miRNAs are gaining recognition for the growing evidence of their involvement in both brain development and neurodevelopmental disorders [3].

In this line, if neurodevelopment is a changing process during the lifespan, the expression of miRNAs involved in this process should be perfectly differentiated in relevant age periods. While

studying the expression of miRNAs in brain tissue is troublesome, those changes could be detected in a less invasive way by analyzing the cerebrospinal fluid (CSF), which embeds the brain, and spinal cord collects information from much of the central nervous system (CNS). MiRNAs can be detected in CSF both free circulating and in extracellular vesicles (EVs), which have been suggested as important signaling entities within the CNS [4].

However, published data regarding the expression of miRNAs in CSF from healthy individuals are very scarce, especially in children. This may be due in part to the difficulty in obtaining samples for analysis since CSF is not commonly obtained from healthy individuals in the clinical routine, the low amounts of research fluid that are obtained from clinical settings, and the methodological issues that this entails [5].

In this work we intend to: 1) define changes in miRNA expression during development in the childhood period, 2) compare the expression of miRNAs in children with other age groups (adults and the elderly) using data from the literature, and 3) establish miRNAs that could serve as housekeeping for analysis in CSF.

MATERIALS AND METHODS

Human CSF samples

Samples and data from patients analyzed in this study were provided by the Basque Biobank (www.biobancovasco.org) and were processed following standard operation procedures with appropriate approval of the Ethical and Scientific Committees (code CEIC E17/40). Fourteen non-hemorrhagic samples from previously healthy children (aged 0-7.4), who went to the emergency department for fever, were acquired (Table S1). All of them had a negative result for all the tests performed, including HIV and hepatitis B and C. The CSF was obtained via lumbar puncture, centrifuged to remove contaminant cells (500 x*g* for 10 minutes) (cleared CSF), aliquoted, and stored at -80°C until its analysis. In addition, aiming to identify differences in the expression of miRNAs during life, our data was compared with that published in adults and elders (Table S2).

RNA extraction

Total RNA was extracted from 200 µL of cleared CSF using the Plasma/Serum RNA Purification kit (Norgen #55000), which have previously demonstrated the best yield under the same conditions [5]. Isolated RNA was eluted in 50 µL of nuclease-free water (Ambion #AM9930). To account for the differences in efficiency of the extraction, the samples were spiked with cel-miR-39 (2×10^{-4} nmoles added) (Invitrogen) after mixing the cleared CSF with cell disruption buffer, to avoid its degradation by RNases.

Small RNA sequencing

Quantity and profiles of the obtained RNAs were evaluated using Agilent RNA 6000 Pico Chips (Agilent Technologies #5067-1513). Then, starting from 40 µL of each extraction, sequencing libraries were prepared with the NEXTflex™ Small RNA-Seq Kit v3 (Bioo Scientific Corp #5132-05), following the protocol V16.06. PCR products were resolved on 6% Novex TBE PAGE gels (Thermo Fisher Scientific #EC6265BOX), and the region between 150 bp and 300 bp was cut from the gel. Small RNAs were extracted from polyacrylamide gel using an adapted protocol, in which DNA from gel slices was diffused in molecular grade water. Obtained libraries were qualified and quantified using Agilent High Sensitivity DNA kit (Agilent Technologies # 5067-4626) and Qubit dsDNA HS DNA Kit (Thermo Fisher Scientific # Q32854), respectively. Libraries were single-read sequenced for 51 nucleotides in a HiSeq2500 (Illumina).

MiRNA expression comparison among age groups

Aiming to identify differences in the expression of miRNAs during life, sequencing data obtained in children were compared with that published in adults and elders (Table S2). The adult group comprised seven healthy individuals with >35 years. Raw counts for each sample were provided by Godoy et al. [6]. Regarding elders, we selected 11 controls over 75 years from Burgos et al. [7] that presented more than 2.8 million reads in their smallRNAseq analyses. Raw counts were obtained through the exRNA Atlas [8] (sample metadata code: EXR-KJENS1sPlvS2-AN). To compare all the studies, raw counts for each miRNA in each sample were normalized dividing by the total number of read counts in that sample [6], i.e. relative abundance.

Data analyses

Small RNA sequencing. FASTQs were trimmed for the adapters following the manufacturer's recommendations of the NEXTflex™ Small RNA-Seq kit. We used bowtie [9] to align reads against the human genome (GRCh38) considering a mismatch=0, to avoid false positives. Quantification using mature miRbase (version 22) was performed by Partek expectation maximization (EM) using the Partek Flow software version 7.0. All the miRNAs that were present in the blank (ultra-pure water that underwent the same processes as the CSF samples) above 1% were eliminated from this study. Only those miRNAs presenting ≥10 counts in at least one sample were considered. Additionally, to avoid bias due to any non-evaluated disease, only miRNAs present in all the individuals of each age-group were considered for comparisons. Data were presented in relative abundance, which was calculated by dividing the number of each miRNA raw counts/sample by the total number of read counts/sample.

Gene targets prediction and pathway enrichment analysis. Predicted target genes for each miRNA were obtained using miRWalk 2.0 database [10]. Only those targets predicted by ≥8 prediction programs of the 12 available in miRWalk 2.0 were considered. Once predicted target genes were identified, pathways enrichment analyses were performed using ConsensusPathDB web tool (CPdB) [11] by considering the collections of BioCarta

(http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways), Reactome [12], and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [13].

RESULTS AND DISCUSSION

The search for biomarkers requires molecules that remain stable in healthy conditions but undergo changes in their expression levels during illness. With this in mind, we studied the CSF microRNAome of 14 children and compared it with that described in adults and elders. We aimed to point out the biological variability of miRNA expression in the CSF throughout life. Getting to know their expression in healthy conditions may help establish new biomarkers of CNS pathologies, especially in children, where they are desperately needed, as well as the identification of novel housekeeping miRNAs for a robust data normalization in this bodily fluid. For that, we used the NOR method described by Prieto-Fernández et al. [5] for miRNA extraction as it seems the most suitable for analyzing both free-circulating and EVs-associated miRNAs using low CSF inputs.

Expression of CSF miRNAs in the first years of life

The human brain size increases rapidly during the first postnatal year to approximately 70% of its adult size [14], being a time of big changes in the brain. Another milestone of early life neurodevelopment occurs around the 3 years of life, the age at which increases in cortical thickness are slowed and oligodendrocytes have been robustly generated and migrated [15–17]. Thus, we separated our cohort of pediatric individuals in three different age-groups: <1 (n=6), 1-2 (n=3), and >3-years (n=5), which included individuals up to 7.4 years. Overall, 536 different miRNAs were identified (the sum of all unique miRNAs detected in our study) (FASTQ data are available in GEO with the accession number GSE148359). The youngest group (<1 year) showed the highest number of miRNAs (n=507) while the 1-2 and >3 years groups harvested a lower diversity (n=155 and n=277, respectively). The number of miRNAs detected in each sample, as well as the results of sequencing and computational post-processing are shown in Table S3. We revealed a high diversity in both number and variety of miRNAs in the first years of life, especially during the first year (Table S3 and S4). This suggests that some miRNAs are expressed only at specific points of the CNS development.

To analyze the expression levels in the common miRNAs during childhood only the extreme age-groups were compared (<1 vs. >3 years), being the intermediate group (1-2 years) omitted to avoid background noise. After applying our detection criteria for each miRNA (≥10 counts in all the individuals within a group), 41 common miRNAs were identified (Table S4).

The differential expression analysis, based on the 41 common miRNAs showed that two miRNAs were differentially expressed between <1 and >3 years groups, i.e. miR-423-5p, overexpressed in the <1 year group and miR-22-3p, overexpressed in the >3 years group (Figure 1A). The relative abundance of these miRNAs in the 1-2 years group was intermediate between the younger (<1 year) and the older (>3 years) groups yet no significant differences were identified

between this intermediate group and the other two groups (<1 and >3 years), only the differences between the extreme groups being statistically significant (Figures 1B and C).
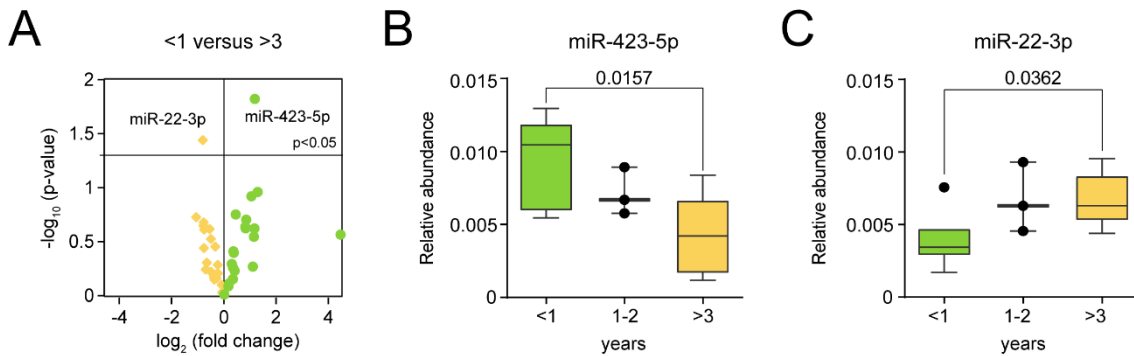

Figure 1. **Expression of CSF miRNAs in the first years of life**. **(A)** Volcano plot of the 41 common miRNAs. A positive $\log_2$ fold change value indicates increased abundance in <1 year group (right, green) and vice versa (left, yellow). All p-values were adjusted using the Bonferroni method. **(B,C)** Boxplots showing the relative abundance of the two miRNAs differentially expressed between <1 and >3 years groups: miR-423-5p (downregulated in >3 years group) (B) and miR-22-3p (downregulated in <1 group) (C), including the individuals of the 1-2 years group. The p-values calculated by the unpaired t-test for each significant comparison are also depicted. Abbreviations: less than 1 (green), from 1 to 2 (black), and more than 3 years (yellow) groups (<1, 1-2, and >3, respectively).

The overexpression of miR-423-5p in the youngest age group (Figure 2B) could suggest a role in neuronal proliferation. In fact, miR-423 has been shown to promote cell growth and cell cycle progression [18] and higher expression of miR-423-5p has been associated with progression in glioma cells [19], while low expression of this miRNA has been observed in neurodegenerative disease [20], which would support this hypothesis. However, these results should be interpreted with caution since miR-423-5p were shown to be upregulated with increasing age in blood from healthy individuals [21].

On the other hand, mir-22 (Figure 2C) would be more relevant in a phase in which proliferation is not that relevant. Indeed, mir-22 has been shown to have an antiapoptotic and neuroprotective effect instead [22–25].

Furthermore, in order to identify miRNAs that were specific of an age group, we considered only those miRNAs that presented ≥10 counts in all the individuals of a specific age group and in less than 20% of the individuals of the group with which it is compared. We identified 4 exclusive miRNAs in the <1 year group (miR-127-3p, miR-30b-5p, miR-487b-3p, and miR-574-5p) and no specific miRNAs in the >3 years group (Table S5). However, to complete a comprehensive miRNA expression map during childhood, further studies analyzing a more accurate range of ages are needed. In addition, these results must be considered in a wider context, considering miRNA diversity during life.

Different miRNAs expression patterns during life

Then, we aimed to unveil the CSF miRNA diversity during life. For that, we compared miRNAs identified in the >3 years group with the previously published data in adults and elders (Table S2), in which, following our defined criteria (≥10 counts in all the individuals of each age-group), 212 and 220 CSF miRNAs were identified, respectively. The comparison between children (>3 years) and adults, revealed 131 common miRNAs. We also compared the adults with the elderly group and found a more robust miRNA stability in ageing since 242 common miRNAs were observed (Table S5).

Next, we calculated the differences in the expression of miRNAs that were found expressed in all age groups. We found 18 miRNAs that were, at least in two comparisons, significantly up or downregulated at different time points considering a p-value of 0.05 (Table 1), which were the ones that we considered for further analyses.

We also compared the correlation between the expression levels of the 18 miRNAs and age (Figure 2).
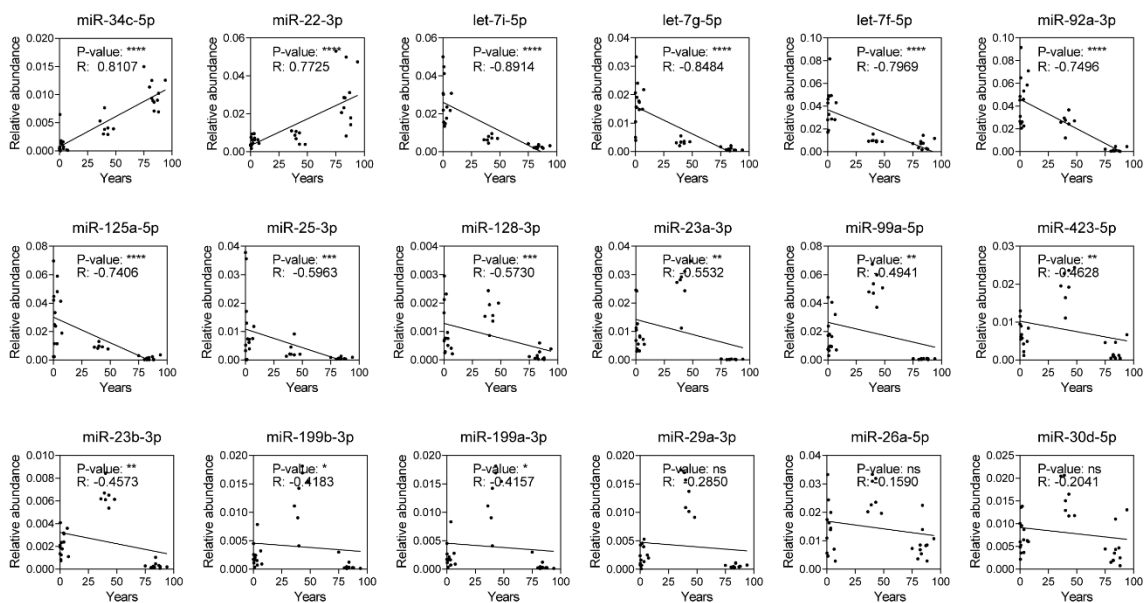

Figure 2. Different miRNAs expression patterns during life. Dot plots showing the linear correlations between relative expression of the 18 common miRNAs that presented differences in expression among age groups and age. The R Spearman correlation coefficient and p-value are provided for each plot. Asterisks represent p-values as follows: ns (P>0.05), * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001), and **** (P ≤ 0.0001).

In the line of the results of the t-tests, we found that two miRNAs, miR-34c-5p and miR-22-3p, presented a strong positive correlation with age and might be relevant for processes related to differentiation and senescence. In fact, mir-22 has been shown to have an antiapoptotic and neuroprotective effects, as mentioned above [22–25], and the miRNAs of the mir-34 family have

been shown to have higher expression in mature and differentiated neurons [26,27] and have been associated with healthy brain aging [28]. On the contrary, six miRNAs (let-7i-5p, let-7g-5p, let-7f-5p, miR-92a-3p, miR-125a-5p, and miR-25-3p) presented a negative correlation with age. All of them, although demonstrating a high variability in the children (Table 1), were upregulated during the first stage of life and may be associated with processes of relevance in this time period, such as proliferation. In fact, these miRNAs have been associated with stemness maintenance [29,30], proliferation [31,32], and some are deregulated in neurodegenerative disorders [33]. The expression dynamics of these eight miRNAs are also depicted in the volcano plots of Figure S1. The rest of them (miR-128-3p, miR-23a-3p, miR-99a-5p, miR-423-5p, miR-23b-3p, miR-199b-3p, miR-199a-3p, miR-29a-3p, miR-26a-5p, and miR-30d-5p), although 7 out of 10 presented a negative correlation (Table 1), it is evident that they reached their highest peak of expression in the adulthood but then decreased in old age (Figure 2). This outcome is in accordance with previous studies suggesting that the elderly (ELD) present a microRNAome more similar to that of children than that of adults (unpublished data).

On the other hand, as many reports reflect gender differences in the pattern of miRNA expression, especially in adults, we compared the expression of these 18 differentially expressed miRNAs between male and female across the three main groups (CHI, ADU, and ELD). We did not find any significant difference on the 2-way ANOVA performed, except for miR-22-3p in the elderly group ($p<0.0001$), which does not support a major effect of age.

Interestingly, we demonstrated the capability of these 18 common miRNAs, which were differentially expressed during life, to discriminate individuals from different age groups (Figure 3). This remarks the complex differential miRNA expression profile that can be observed through life. It must be noted that these findings are also of relevance for the study of neurological pathologies. We sought for references that described these 18 miRNAs as biomarkers without considering the biofluid that contains them, based on MDD v3.0 database (http://www.cuilab.cn/hmdd) [34]. We found that 16 out of 18 miRNAs had been proposed as diagnostic (Table S6) or prognostic (Table S7) biomarkers in 67 different diseases, including Alzheimer disease, child development disorders, different types of lymphoma, and cancer. In this context, these outcomes underline the need of including appropriate age-matched controls when the expression of miRNAs is analyzed in different pathological contexts; otherwise, differences in expression associated with the disease could be affected by differences due to age. This is of special interest for ageing research and in pediatric malignancies, in which adult controls are commonly used [35–38]. In addition, some studies have linked the expression of miRNAs in blood or serum with changes that take place during ageing [33,39,40], and this could be a less invasive alternative. However, further research is needed since different biofluids may lead to different circulating miRNA patterns [6].

On the other hand, we also identified a list of exclusive CSF miRNAs that were specific of an age group (Table S8). These age-specific miRNA signatures were used to predict the target genes and the related pathways in which they might be involved employing the default collections of

KEGG, Reactome, and BioCarta (Table S9 to S12). The common predicted pathways among age-specific miRNA signatures are depicted in Figure S2. On the contrary, all the specific pathways predicted for each age-dependent miRNA signature are listed in Table S13.
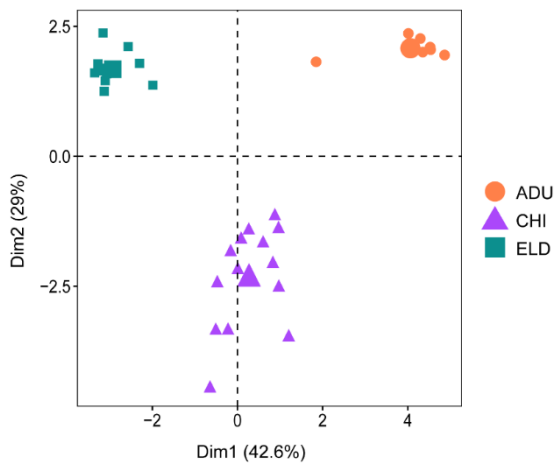

Figure 3. The 18 miRNAs were able to discriminate individuals from different age groups. Principal Component Analysis (PCA) of the complete cohort considering the expression (relative abundance) of the 18 common miRNAs presenting significant differences between children (CHI), adult (ADU), and elder (ELD) groups. The largest mark in each group represents the barycenter.

Potential housekeeping miRNAs in CSF

The smallRNAseq data normalization remains a hot topic that still needs to be addressed, especially considering the variability in the expression of some miRNAs observed in different age periods. Until now, several miRNAs have been proposed to normalize data in experiments involving CSF [5]. However, none of them has shown stable levels in all pathological conditions and CSF components [41]. In this study, we have demonstrated that, even in healthy conditions, CSF miRNAs present variable expression levels throughout life, which is more noticeable during first years after birth. For that reason, it is crucial to define molecules that did not change their expression throughout the life of the individual.

With that in mind, we sought miRNAs showing non-significant differences between age-groups (Table 2). We detected four miRNAs (miR-92a-3p, lef-7f-5p, let-7i-5p, and miR-30d-5p) presenting low variability during infancy (i.e. common miRNAs showing a standard deviation/relative abundance ratio below 0.4) (Figure 4A). These miRNAs may serve as potential house-keepings during this life period (from birth to 7.4 years old).

Additionally, one miRNA, miR-191-5p, was not differentially expressed among all age-groups tested (Figure 4B). However, it presented a huge standard deviation during the first year of life (0.0087 ± 0.0097). Therefore, it could only be considered as housekeeping after the first year of life.

In view of a lack of stable CSF miRNAs lifelong, using a combination of several non-significant miRNAs presented herein (according to the age of the individuals analyzed) could be a valuable approach for further studies (Table 2). However, greater efforts are needed to discover robust and age-dependent CSF house-keeping miRNAs to normalize smallRNAseq data in the future.
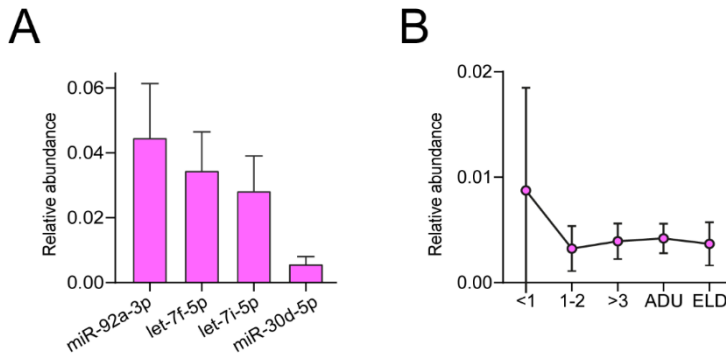

Figure 4. Potential housekeeping miRNAs in CSF. (**A**) mean relative abundance and standard deviation of the four miRNAs presenting the lowest variability during infancy in this study (N=14). Only the miRNAs exhibiting a standard deviation/mean ratio below 0.4 were considered. (**B**) Spaghetti plot of the relative abundance (y-axis) of miR-191-5p, a potential housekeeping for its use from 1 year to death. <u>Abbreviations</u>: less than 1, from 1 to 2, and more than 3 years groups (<1, 1-2, and >3, respectively); Adult group (ADU); Elderly group (ELD).

LIMITATIONS OF THE STUDY

We are aware of the limitations of our study in terms of number of samples per age-group. On the one hand, the availability of CSF of healthy infants and children is scarce in volume as well as in number of samples available for research. On the other hand, the number of studies analyzing the microRNAome of CSF samples in healthy adults and elders to compare with is limited. This is usually a common problem in studies involving CSF that generally analyze a similar number of controls than in this work [6,42–44]. Another limitation could be that, although the patients were negative for HIV and hepatitis B and C, and the clinical parameters analyzed were within normality, we cannot exclude that the feverish process of the patients could have affected the expression of the miRNAs. For all these reasons, and even though our data should be interpreted with caution, we consider that our outcomes could be relevant for the community and would foster more investigation in this field, which may help to decode the biological variability in this bodily fluid.

TABLES

Table 1. A list of the 18 miRNAs presenting significant differences between children (CHI), adult (ADU), and elder (ELD) groups (unpaired t-test, p<0.05). Arrows indicate if the miRNA is significantly up (↑) or down (↓) regulated in the youngest group of each comparison. P-values that remained significant after Bonferroni correction were marked in bold. The R coefficient and p-value of each Spearman correlation between the relative expression of the 18 miRNAs and age are also provided. <u>Abbreviations</u>: Regulation (Reg).

MiRNA	CHI vs. ADU		ADU vs. ELD		CHI vs. ELD		Spearman correlation	
	Bonferroni p<0.0014	Reg	Bonferroni p<0.0003	Reg	Bonferroni p<0.0016	Reg	R	P
miR-34c-5p	**9.50E-04**	↓	**2.19E-05**	↓	**1.05E-08**	↓	8.11E-01	<0.0001
miR-22-3p	NS		6.95E-04	↓	**3.36E-04**	↓	7.73E-01	<0.0001
let-7i-5p	**1.52E-05**	↑	**1.16E-04**	↑	**2.05E-06**	↑	-8.91E-01	<0.0001
let-7g-5p	**1.31E-05**	↑	3.45E-04	↑	**2.29E-06**	↑	-8.48E-01	<0.0001
let-7f-5p	**1.83E-05**	↑	2.34E-02	↑	**3.74E-06**	↑	-7.97E-01	<0.0001
miR-92a-3p	8.18E-03	↑	**9.16E-05**	↑	**3.46E-06**	↑	-7.50E-01	<0.0001
miR-125a-5p	1.96E-03	↑	**7.14E-07**	↑	**1.36E-04**	↑	-7.41E-01	<0.0001
miR-25-3p	3.08E-02	↑	4.24E-02	↑	5.14E-03	↑	-5.96E-01	3.00E-04
miR-128-3p	NS		**1.33E-04**	↑	2.31E-03	↑	-5.73E-01	6.00E-04
miR-23a-3p	**3.83E-04**	↓	**8.87E-05**	↑	**1.98E-04**	↑	-5.53E-01	1.00E-03
miR-99a-5p	**4.75E-06**	↓	**8.55E-06**	↑	**3.52E-04**	↑	-4.94E-01	4.00E-03
miR-423-5p	**1.24E-04**	↓	**1.9E-05**	↑	**2.17E-04**	↑	-4.63E-01	7.60E-03
miR-23b-3p	**2.57E-07**	↓	**1.18E-06**	↑	**1.53E-05**	↑	-4.57E-01	8.50E-03
miR-199b-3p	**1.08E-03**	↓	5.77E-04	↑	7.68E-03	↑	-4.18E-01	1.72E-02
miR-199a-3p	**1.04E-03**	↓	5.77E-04	↑	1.12E-02	↑	-4.16E-01	1.80E-02
miR-29a-3p	**5.40E-05**	↓	**5.80E-05**	↑	3.18E-03	↑	-2.85E-01	1.14E-01
miR-26a-5p	1.33E-03	↓	**4.17E-05**	↑	NS		-1.59E-01	3.85E-01
miR-30d-5p	**4.43E-04**	↓	**4.66E-05**	↑	NS		-2.04E-01	2.63E-01

Table 2. List of miRNAs showing, at least, two non-significant (NS) values between all individuals of the groups considered. They could serve as normalizers in further studies. Significant p-values (unpaired t-test) >0.05. <u>Abbreviations</u>: less than 1 and more than 3 years groups (<1 and >3, respectively); Adults (ADU); Children (CHI); Elderly (ELD); Not significant (NS).

miRNA	P-values (t-test)				
	<1 vs. >3	>3 vs. ADU	CHI vs. ADU	ADU vs. ELD	CHI vs. ELD
miR-191-5p	NS	NS	NS	NS	NS
miR-126-3p	NS	NS	NS		
miR-150-5p	NS	NS	NS		
miR-223-3p	NS	NS	NS		
miR-204-5p	NS	NS	NS	2.07E-11	1,07E-13
miR-10a-5p	NS	NS	NS	5.51E-05	1,34E-03
miR-24-3p	NS	NS	NS	2.59E-04	2,93E-06
let-7e-5p	NS	NS	NS	2.86E-04	9,84E-04
miR-335-5p	NS	NS	NS	6.41E-04	7,82E-04
let-7d-3p	NS	NS	NS	1.34E-03	5,27E-06

miR-143-3p	NS	NS	NS	3.05E-03	3,70E-05
miR-99b-5p	NS	NS		2.35E-03	
miR-423-3p	NS	NS		1.02E-02	
miR-27a-3p	NS	NS	1,66E-01	6.37E-03	4,87E-04
miR-10b-5p	NS	NS	1,96E-02	2.71E-04	1,17E-03
miR-100-5p	NS	NS	1,30E-02	3.51E-05	1,15E-02
let-7d-5p	NS	NS	5,96E-04	1.63E-05	2,97E-06
miR-451a	NS	2.14E-02	2,40E-02	NS	1,95E-02
miR-30a-5p	NS	1.22E-02	8,44E-03	NS	1,59E-02
miR-1298-5p	NS	1.14E-04		NS	
miR-93-5p		NS		NS	
miR-197-3p		NS		NS	
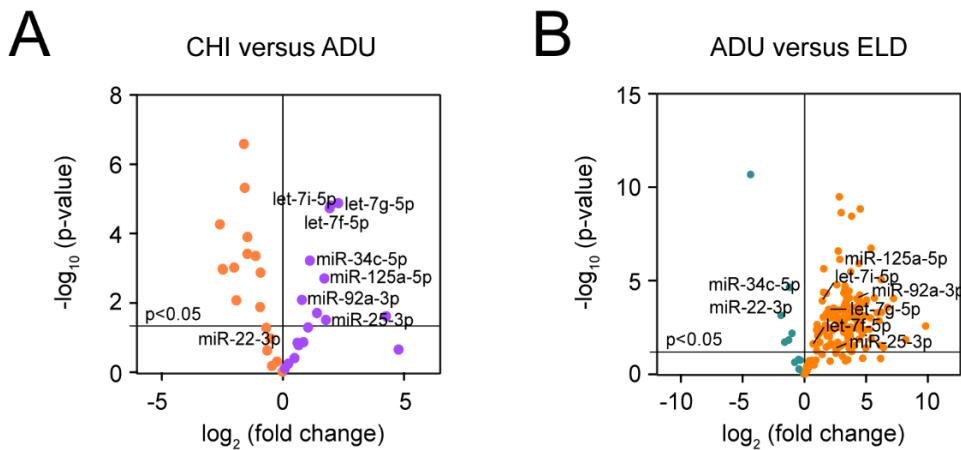
Figure S1. (A,B) Volcano plot of the common miRNAs between children (CHI, purple) and adults (ADU, orange) (A), as well as between adults (ADU, orange) and elders (ELD, green) (B). A positive log_2 fold change value indicates increased abundance in the youngest group. Only the labels of the eight miRNAs that reached their peak of expression in childhood or the elderly group are indicated (see Figure 2 and Table 1). All p-values were adjusted using the Bonferroni method.
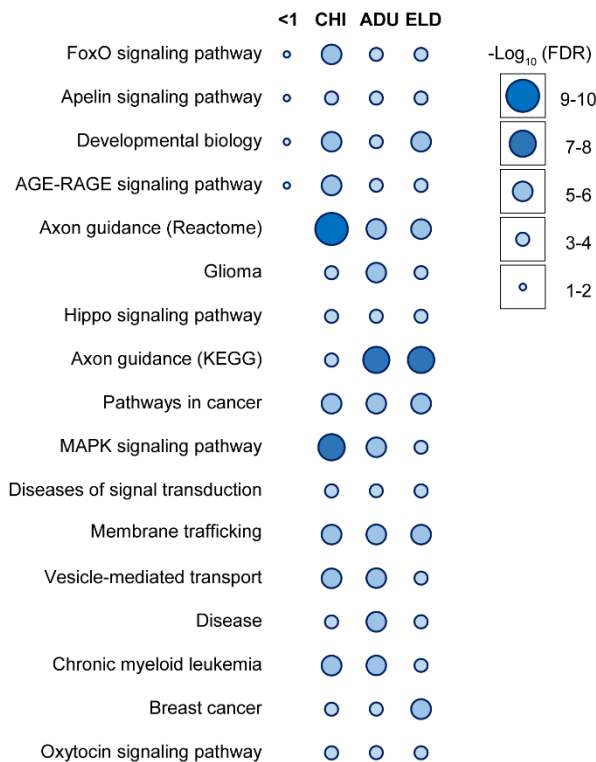

Figure S2. Common predicted pathways among the different age-specific miRNA signatures. FDR values are indicated in negative logarithmic scale (-log_{10}), therefore, the highest value represents the most statistically significant predictions. Abbreviations: less than 1 year group (<1); Adults (ADU); Children (CHI); Elders (ELD).

ABBREVIATIONS

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Less than 1, from 1 to 2, and more than 3 years groups (<1, 1-2, and >3, respectively); ADU: adult group; CHI: children (individuals from 0 to 7.4 years old); CSF: cerebrospinal fluid; CPDB: ConsensusPathDB web tool; ELD: elderly group; EVs: extracellular vesicles; KEGG: Kyoto Encyclopedia of Genes and Genomes; mRNA: messenger RNA; miRNA: microRNA; RT-qPCR: quantitative PCR; smallRNAseq: small RNA sequencing.

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