Received: 10 May 2023 | Revised: 21 July 2023 | Accepted: 20 September 2023

DOI: 10.1111/gbb.12868

ORIGINAL ARTICLE

Genes, Brain and Behavior

Extracellular vesicles from cerebrospinal fluid revealed changes in miR-19a-3p and miR-4516 expression in Slovene male suicides

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Funding information
Javna Agencija za Raziskovalno Dejavnost RS, Grant/Award Numbers: Research Programme P1-0390, Research Project Z3-2653

Abstract

Suicide is an important public-health concern, with more than 700,000 people dying by suicide yearly. It is a multifactorial phenomenon, shaped by the effects of sociodemographic, environmental and biological factors. The latter two factors can be linked through epigenetic studies, which examine differences in gene expression that are not due to changes in the DNA sequence itself. Epigenetic mechanisms include micro RNAs (miRNAs), which have a direct effect on already translated mRNA, leading to either decay or translational repression of the target mRNA. MiRNA molecules have been identified as cargo of extracellular vesicles (EVs) used by cells for long-distance communication, and pathophysiological changes in miRNA in brain cells may be reflected in cerebrospinal fluid (CSF) vesicles. In this study we investigated the presence and differential expression of selected miRNAs in EVs from the CSF of male suicide completers and controls. Western blot and nanoparticle tracking analyses confirmed the presence of small and medium sized EVs. Of the miRNA analyzed (miR-16-5p, miR-19a-3p, miR-34c-5p, miR-17-5p, miR-4286, miR-26b-5p, miR-381-3p, and miR-4516) miR-19a-3p and miR-4516 reached statistical significance with *p*-values of 0.0408 and 0.0168, respectively. Mir-4516 and miRNA-19a-3p have been previously studied in suicide, and target *SLC6A4* and *TNF-α* expression, correspondingly. Approximately 70% of known miRNAs are expressed in the central nervous system, and therefore represent an important biomarker potential. Investigating the cargo of CFS and blood EVs would further support the identification of miRNAs with clinical use potential.

KEYWORDS
CSF, epigenetics, EVs, micro RNA, miR-19a-3p, miR-4516, miRNA expression, Slovene male suicides, suicide, suicidal behavior

1 | INTRODUCTION

1.1 | Suicide

According to the World Health Organization, more than 700,000 people die because of suicide every year.[1] Slovenia ranks among countries with the highest suicide rate (number of suicides/100,000 citizens) in Europe and globally.[2] The ratio between male and female completed suicides is different among countries but overall suicide rate is 2.3 times higher in males than in females, as reported by World Health Organization.[3] The most common global methods for suicide are pesticide self-poisoning, firearms, and hanging, the last is the most common method also in Slovenia.[1,2]

Understanding suicidality is difficult due to its complex multifactorial nature, so a multidisciplinary approach is needed. There are many different risk factors for developing suicidal behavior that could be designated as distal and proximal factors. Distal factors include family history, early-life adversity, personality traits like high impulsiveness, genetic, and epigenetic changes. Proximal factors include mental disorders and other illnesses, molecular changes, and acute substance misuse. Distal factors can increase suicide risk, and proximal factors are more directly affecting suicidal behavior.[4]

From studies made on twins and adopted children, it was estimated, that there is around 30%–50% genetic contribution to suicidal behavior. Genetic studies of the suicide phenomena have provided some evident findings of the association between single nucleotide polymorphisms and suicide.[5] In the last 20 years, also studies of epigenetics are growing and gaining importance in the field of psychiatry.[5,6]

Researchers are investigating different functional pathways to understand suicide and suicidal behavior.[7] Many studies investigated serotonin pathway and demonstrated dysregulated transmission in brain tissue and cerebrospinal fluid (CSF).[8–11] The pathogenesis of suicide involve also dysfunction of hypothalamic–pituitary–adrenal (HPA) axis and polyamine stress response. Expression of neurotrophic genes is dysregulated in brains of suicides, changes were evident also for glutamatergic and GABAergic dysfunction. Moreover, several evidence are suggesting also a link between inflammation and suicidal behavior.[11,12]

1.2 | Micro RNAs

The term epigenetics refers to chemical and physical modifications of the genome without changing DNA sequence. Three most studied epigenetic modifications are DNA methylation, histone posttranslational modifications, and non-coding RNAs. DNA methylation and histone posttranslational modifications regulate DNA transcription to messenger RNA (mRNA). On the other hand, micro RNAs (miRNAs), one of the non-coding RNAs, regulate translation from mRNAs into proteins.[13]

MiRNAs are 19–24 nucleotides (nt) long RNAs formed in multiple steps of biogenesis. In the first step, RNA polymerase II transcribes 1 kb long primary miRNA. Primary miRNA has a stem and a loop structure. In the second step, Drosha ribonuclease III cleaves primary miRNA into a 60–100 nt long precursor miRNA. Exportin 5 translocates precursor miRNA from the nucleus into the cytoplasm. There, Dicer ribonuclease cleaves the loop structure into a ~22 nt long unstable, mature, double stranded RNA. One strand of the double stranded RNA is degraded and the other becomes the mature miRNA. Nearly always, the strand with lower stability at the 5′ terminus becomes the 'guide strand', which is incorporated into the RNA-induced silencing complex (RISC) along with Argonaut proteins. This miRNA-RISC (miRISC) directs the complex to the target mRNA. MiRNA sequence is complementary to one or more mRNAs.[14,15] The impact of the miRNAs binding to the mRNAs results either in decay or in translational repression of the target mRNA. The outcome is influenced by the degree of complementarity between miRNA and mRNA sequences and the location of the miRNA-binding site. Full complementarity results in the degradation of both molecules.[15]

Studies in suicides showed altered miRNA expression of several miRNAs.[16,17] MiRNA wide approach studies[18–20] and candidate approach[21–25] were used to show differentially regulated miRNAs. However, psychiatric diagnosis (such as major depressive disorder) and suicide often co-occur, therefore it is harder to conclude if the observed difference is attributed to the suicide or other psychopathologies.[16,17]

1.3 | Extracellular vesicles

Extracellular vesicles (EVs) are a group of cell-derived vesicles produced by most cells, and found also in biofluids. They transport miRNAs, DNA, proteins, and lipids between cells. According to the size and biogenesis, EVs are classified into three groups. Apoptotic bodies (50–5000 nm) and microvesicles (100–1000 nm) that bud off from the plasma membrane, and the third, exosomes (30–150 nm), intraluminal vesicles that are developed with inward budding of the multivesicular body. Multivesicular bodies can be then fused with the plasma membrane, and exosomes are released into the extracellular space.[26,27]

One way of miRNA transportation around body are miRNAs packed in EVs.[28] In mental disorder studies, several miRNAs were identified as possible biomarkers.[16,29] However, there is a lack of studies investigating miRNAs from EVs of the subjects with mental disorders, a promising source of RNA biomarkers.[5,16] EVs in central nervous system serve as a communicating system. They are secreted by different cells and carry message between neighbor and more distal cells – neurons, glia, and other cell types.[30] Pathophysiological changes of the miRNAs in brain cells are mirrored also in the EVs content where a real-time status of the brain is reflected.[30,31] EVs are secreted into the CSF that is in direct contact with central nervous system and therefore contains information from brain tissue.[30] It has an important function, as mechanical protection and nourishment – waste disposal and intercellular communication.[32] EVs, in particular exosomes, have an ability to cross the blood–brain

barrier, which is opening a window for a non-invasive way of exploring brain status through the exosome analysis in the blood plasma.[30,33,34]

To date, there is no study investigating miRNAs from EVs of the CSF in suicides, since so far miRNAs were investigated in specific candidate gene approach or broader investigating miRNA expression across the genome.[16] Studies were made on prefrontal cortex or blood and in some cases on CSF. The aim of our study was to investigate the presence and differential expression of selected miRNAs in EVs from the CSF of suicides compared to a control group. MiRNAs included in this study were differentially expressed in the published overviewed articles of the depression and suicide.

2 | MATERIALS AND METHODS

2.1 | Sample collection and subjects

In our study, we included 40 CSF samples of male suicides who died by hanging and males who died due to cardiac arrest, as a control group. The autopsies were performed at the Institute of Forensic Medicine, Faculty of Medicine, University of Ljubljana. For a purpose of this study, we obtained an agreement to collect samples from the National Medical Ethics Committee of the Republic of Slovenia (0120-520/2019/4). Besides the cause of death, our selection criteria were sufficient body preservation and age up to 65 years to avoid potential age related neurodegeneration. The body is sufficiently preserved when post mortem interval is less than 36 h or if body is without late post mortem changes, such as signs of decomposition, for example; autolysis and putrefaction: greenish discolouration of the skin, marbling (greenish black coloration along vessels, bloating, vesicle formation, skin slippage and hair slippage, decomposition fluid draining from mouth and nose or accumulation in body cavities).

CSF samples were collected during routine course of autopsy from *cisterna magna* with a puncture through atlanto-occipital membrane and held on ice until storage at −80°C. The average age ± standard deviation (SD) of the suicides group was 42.8 years ±12.7, and of the control group 55.5 years ±7.1. The average post mortem interval ± SD of the suicides group was 29.2 h ±13.3 and of the control group 26.3 h ±21.6. Table with information of each subject including toxicology testing can be found in Supplementary.

2.2 | Extracellular vesicles isolation

Samples were thawed in the fridge at 4°C overnight. All centrifugations were held at 4°C. Firstly, 4–5 mL of the sample was centrifuged at 2000 × *g* for 15 min, and the supernatant was transferred into a fresh tube and centrifuged at 10,000 × *g* for 20 min. With the first two steps of centrifugation, we cleaned CSF for some possible cell debris. Next, 4 mL of the supernatant was transferred into the 5 mL tube for ultracentrifugation. Samples were ultracentrifuged at 100,000 × *g* (swinging rotor MLS-50; Beckman Coulter, Brea, CA, USA) for 100 min. Next, supernatant was discarded and the pellet was resuspended with 1 mL of the cold PBS and transferred into 1.5 mL tube for second ultracentrifugation at 100,000 × *g* (TLA-55 rotor; Beckman Coulter, Brea, CA, USA) for 90 min. The supernatant was discarded and the pellet was resuspended in 50 µL of cold PBS. Each sample was stored in two tubes, each containing 25 µL of the sample. Isolated EVs were stored at −80°C.

2.3 | Extracellular vesicles characterization

We characterized isolated EVs to prove successfulness of the isolation method. Following Minimal Information of Extracellular Vesicle Studies 2018, EVs in our study are referred to as a mixture of small (<200 nm) and medium/large (>200 nm) EVs.[35] For EVs characterization, we used two methods, Western blot (WB) analysis, and nanoparticle tracking analysis (NTA).

Before WB analysis, we measured protein concentration with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on 10 µL of the sample. The rest, 15 µL of the sample was denatured and loaded onto a NuPAGE 4%–12% Bris-Tris gel. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked in PBS with 5% milk for 1 h at room temperature. Primary antibodies - rabbit anti-flotillin-1 (1:1000; Cell Signaling Technology, USA), mouse anti-HSP70 (1:200; Santa Cruz, Dallas, TX, USA), mouse anti-CD9 (1:200; Santa Cruz, Dallas, TX, USA), and rabbit anti-Calnexin (1:2000; Sigma Aldrich, St. Louis, Misuri, USA) - were diluted in PBS with 1% milk and incubated overnight at 4°C with PVDF membrane. The PVDF membrane was washed in 0.1% PBS-Tween, incubated with secondary antibodies - goat anti-rabbit HRP-conjugated antibody (1:7500; Sigma Aldrich, St. Louis, Misuri, USA), and goat anti-mouse HRP-conjugated antibody (1:4000; Sigma Aldrich, St. Louis, Misuri, USA) diluted in PBS with 1% milk - for 2 h at 4°C, and washed in 0.1% PBS-Tween. PVDF membrane was treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientifc Waltham, MA, USA). Chemiluminescence signals were visualized with iBright FL1500 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) (Figure 1).

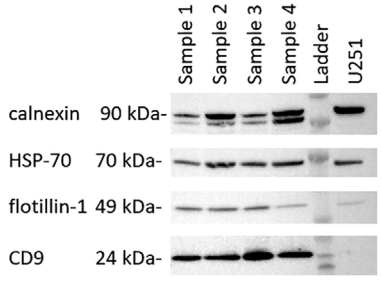

FIGURE 1 WB of CFS' EVs (Sample 1–4) and cell lysate from U251, glioblastoma cell line as a control. Antibodies against proteins enriched in small EVs (HSP70, flotillin-1, and CD9), and against proteins of the endoplasmic reticulum (calnexin), were used.

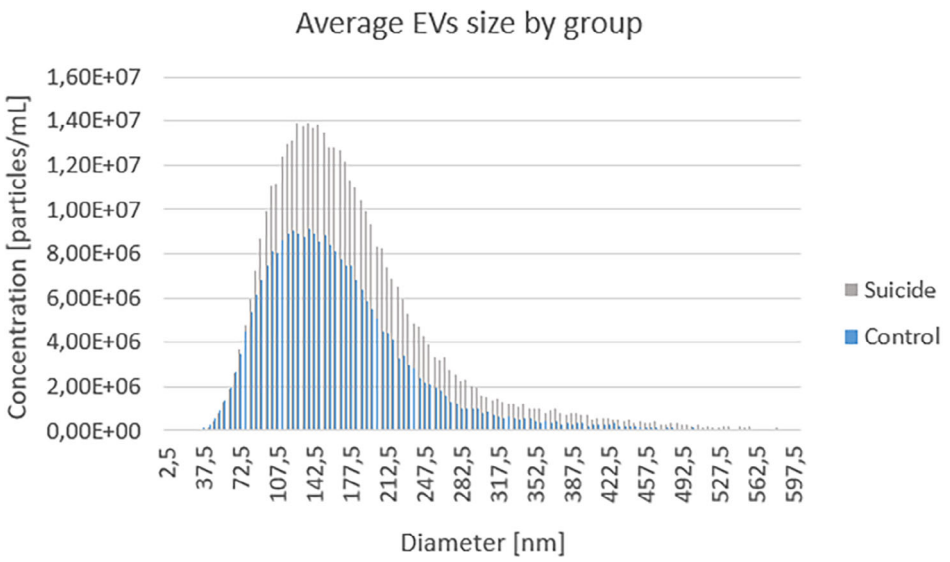

FIGURE 2 Graphical presentation of the size of isolated EVs from NTA.

To detect EVs' size, we used nanosight N300TM (Malvern Panalytical, Malvern, UK) equipped with a syringe pump and autosampler. Isolated EVs were diluted 1:1000 in sonicated PBS to reach optimum concentration range of 1×10^7–10^9 particles. For each sample, six 60-s videos were filmed at camera level 14 and threshold 3. The temperature was set at 25°C. The slide shutter was at 1259 and the slider gain at 366. For detection and analysis, we used software NTA 3.3.- Sample Assistant Dev Build 3.3.203 (Malvern Panalytical, Malvern, UK). For the analysis, four out of six videos were taken for each sample. Results of the particles size were graphically presented (Figure 2).

2.4 | Isolation and gene expression of miRNAs

Total RNA was isolated with *mir*Vana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) from 25 µL of the isolated vesicles, following the manufacturer's instructions. For all samples, 4.84 ng of the isolated RNA was transcribed into cDNA following the protocol from the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). With the last step of the RNA into cDNA transcription, we specifically enriched miRNAs. The qPCR was performed with ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using the hydrolysis probes (Thermo Fisher Scientific, Waltham, MA, USA).

The miRNAs were selected based on published articles overview. We included miRNAs associated with suicide and/or depression (Table 1). From 16 candidate miRNA hydrolysis probes (Table 1), 8 did not show any amplification and were excluded from further qPCR analysis. The remaining 8 miRNA hydrolysis probes were further tested between both groups. Reference miRNAs, that we used for normalization, were selected based on the CSF miRNAs study report.[36] Their stability was calculated with the NormFinder,[37] which showed that the best reference is combination of miR-23a-3p and miR-125a-5p (stability value = 0.142) for normalizing miRNAs of the CSF samples. As such, the two miRNAs, miR-23a-3p and miR-125a-5p, were used as a reference for normalization.

2.5 | Statistical analysis

For each candidate miRNA, expression data were normalized using geometric averaging.[49] For further analysis and figures, we used GraphPad Prism 9. We identified outliers and removed them. Data set distribution was assessed using the D'Agostino and Pearson test. To compare miRNA expression between the suicides and the control group, normal distributed data were analyzed with a t-test, otherwise nonparametric Mann–Whitney U test was used. As statistical significant value, we considered *p*-values <0.05. This is an exploratory study, where we tested if with depression or suicide associated miRNAs are differentially expressed in EVs of the CSF, therefore multiple test correction was not performed.

3 | RESULTS

3.1 | EVs characterization

All 40 subjects (20 suicides and 20 controls) went through the same protocol of EVs isolation. WB analysis was done on 14 samples, which were isolated in duplicates (8 suicides and 6 from control group), as a proof of isolation method. For NTA all 40 samples were used.

Figure 1 represents 4 EVs samples (Sample 1–4) from 4 different subjects, and U251, glioblastoma cell line as a control. Small EVs markers HSP70, flotillin-1, and CD9 were detected, while signal for calnexin indicates presence of medium/large EVs. Marker CD9 is not present in U251 cell line because it is specifically characteristic for EVs. The size of EVs was determined by NTA. Results of the particles size distribution is presented on Figure 2. Average EVs size of the suicides group was 176.8 nm ± SD 11.6, and of the control group was

TABLE 1 List of hydrolysis probes included in the study and published references of miRNAs in human samples.

MiRNA probe	Assay	Comment	Disease state	Tissue	Source
hsa-miR-23a-3p	478532_mir	Reference	/	CSF	36
hsa-miR-125a-5p	477884_mir	Reference	/	CSF	36
hsa-miR-16-5p	477860_mir	Included in the study	MDD	Blood serum CSF and blood	38,39
hsa-miR-19a-3p	479228_mir	Included in the study	Suicide	PBMC	22
hsa-miR-34c-5p	478052_mir	Included in the study	Depressed suicides, MDD	Brain tissue (BA44), Peripheral blood leukocytes	21,40
hsa-miR-17-5p	478447_mir	Included in the study	Subthreshold depression, MDD depressed suicides	Blood serum, Brain tissue (BA10), Brain tissue (LC)	19,20,41,42
hsa-miR-4286	478096_mir	Included in the study	Bipolar disorder died by suicide	Brain tissue (BA24)	23
hsa-miR-26b-5p	478418_mir	Included in the study	MDD, late life depression	PBMC, dlPFC	43,44
hsa-miR-381-3p	477816_mir	Included in the study	Suicide	Brain tissue (BA10)	45
hsa-miR-4516	478303_mir	Included in the study	Suicide	Brain tissue (BA10)	45
hsa-miR-139-5p	478312_mir	Not detected	MDD	Blood EVs, CSF	33,46
hsa-miR-1202	478632_mir	Not detected	MDD, depression	Blood serum, Ventrolateral PFC	38,47
hsa-miR-20b-5p	477804_mir	Not detected	Depressed suicides	Brain tissue (LC)	20
hsa-miR-330-3p	478030_mir	Not detected	Depressed suicides	Brain tissue (LC)	20
hsa-miR-185-3p	478732_mir	Not detected	Suicides with mental disorder	Brain tissue (BA10)	24
hsa-miR-582-5p	478166_mir	Not detected	Depressed suicides	Brain tissue (LC)	20
hsa-miR-218-5p	477977_mir	Not detected	MDD	Brain tissue (PFC)	48
hsa-miR-326	478027_mir	Not detected	MDD with suicide	Brain tissue (rostroventral midbrain)	25

Abbreviations: BA10, Brodmann area 10; BA24, Brodmann area 24; BA44, Brodmann area 44; CSF, cerebrospinal fluid; dlPFC, dorsolateral prefrontal cortex; EVs, extracellular vesicles; LC, *locus coeruleus*; MDD, major depressive disorder; PBMC, peripheral mononuclear cells; PFC, prefrontal cortex.

163.9 nm ± SD 17.3. The main peak for both groups was between 80 and 250 nm. The average concentration of particles/mL was 4.18 $\times 10^8$ ± SD 2.36 $\times 10^8$ for the suicides, and 2.66 $\times 10^8$ ± SD 1.47 $\times 10^8$ for the control group. With this, we conclude there is a big part of small EVs (<200 nm) and a portion of medium/large EVs (>200 nm) in our samples.

3.2 | MiRNA gene expression

Relative gene expression data of eight selected miRNAs were compared between the suicide and control group. Two miRNAs showed statistical significance, miR-19a-3p ($t(14.86) = 2.24$, p-value = 0.0408) and miR-4516 ($t = 42$, p-value = 0.0168), both with lower expression in suicides. Statistical information is presented in Table 2, and graphical presentation in Figure 3.

4 | DISCUSSION

To our knowledge, there is no study investigating miRNAs from CSF EVs in association with suicide. We found two miRNAs, miR-4516 and miR-19a-3p, with different expression in EVs of the CSF when comparing suicides with a control group. Moreover, we found that not all miRNAs are enriched in EVs or are they in such a low amount that cannot be detected with qPCR.

For purpose of this study, 16 miRNAs were selected based on literature screening. All miRNAs were associated with suicide and/or depression in blood, CSF or brain tissue. However, half of miRNAs selected in this study were not detected in EVs isolated from the CSF (Table 1) and this indicates active sorting of EVs cargo. Furthermore, there is growing evidence that the miRNA profile of EVs differs from profile of parent cell.[28] This might explain our contrary results when comparing them with other research studies, particularly those on the brain tissue.

Eight miRNAs (miR-16-5p, miR-19a-3p, miR-34c-5p, miR-17-5p, miR-4286, miR-26b-5p, miR-381-3p, and miR-4516) were tested for further relative expression analysis. Two miRNAs, miR-4516 and miR-19a-3p, showed a significant decrease in expression between the suicide and the control group (Figure 3).

MiR-4516 was proposed to be associated with suicide in our previous research, where the designed algorithm proposed its targeting of the *SLC6A4* gene.[45] *SLC6A4* gene was associated with suicidality in many studies, investigating its expression, DNA methylation, and different gene variants.[8,50,51] Lightly increased expression ($p = 0.0998$) in the Brodmann area 10 of the suicides was detected, but without difference in *SLC6A4* gene expression.[45] Results from present study showed decreased miR-4516 in EVs from the CSF of the suicides indicating there might be alteration in miR-4516 expression, which is not present in Brodmann area 10 but might be in other brain regions.

In one study, expression of miR-19a-3p was investigated in peripheral blood mononuclear cells and dorsolateral prefrontal cortex

TABLE 2 Relative expression of selected miRNAs in EVs from CSF and their *p*-values.

Selected miRNA	Suicides median/mean of relative expression	Control group median/mean	Significance	*p*-value
miR-16-5p	0.01108, $n = 19$	0.004364, $n = 13$	ns	0.0542
miR-19a-3p	0.03495, $n = 17$	0.06108, $n = 13$	*	0.0408
miR-34c-5p	0.01618, $n = 17$	0.01554, $n = 15$	ns	0.7375
miR-17-5p	0.02593, $n = 17$	0.03834, $n = 11$	ns	0.4869
miR-4286	0.08707, $n = 18$	0.1218, $n = 13$	ns	0.5941
miR-26b-5p	0.04639, $n = 18$	0.07481, $n = 16$	ns	0.2811
miR-381-3p	0.1436, $n = 15$	0.5531, $n = 9$	ns	0.0955
miR-4516	0.03476, $n = 14$	0.3507, $n = 13$	*	0.0168

Note: For all miRNAs, except miR-19a-3p, we used Mann–Whitney U test, for which t-test was used.

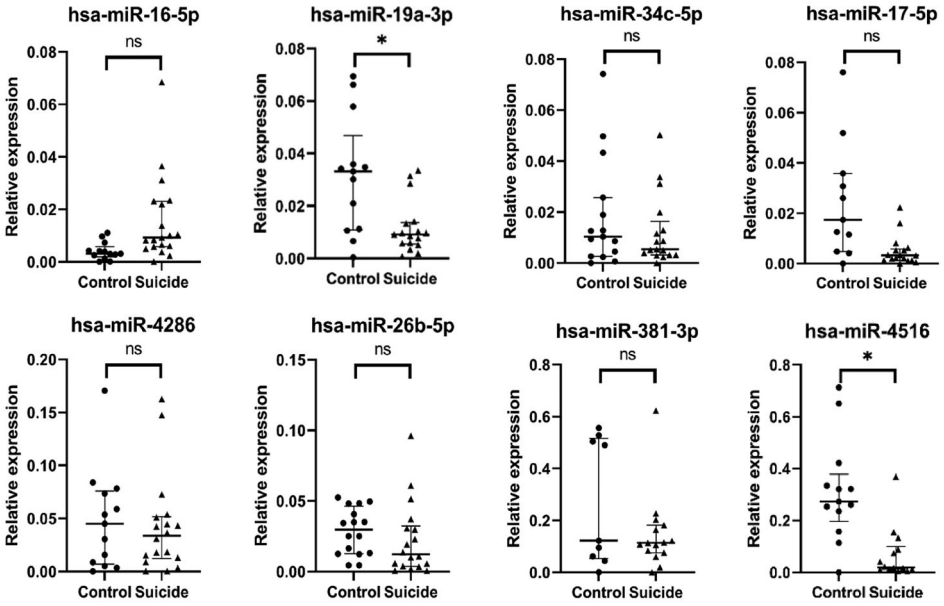

FIGURE 3 Relative gene expression of selected miRNAs from CSFs' EVs in the suicides and control group. Results are presented as a scatter plot. Each point represents a measure of relative gene expression per subject. Horizontal line represents median value ± SD.

of suicides with either major depressive disorder (MDD) or other mental disorders, and a control subjects without mental disorders. In the same research study, they showed the ability of miR-19a-3p to target tumor necrosis factor (TNF)-α, a main proinflammatory cytokine, in in vitro conditions. The results of miR-19a-3p showed its higher expression in the suicide group with MDD, compared to non-suicide MDD subjects in dorsolateral prefrontal cortex. In peripheral blood mononuclear cells miR-19a-3p was increased in MDD patients with serious suicidal ideation. The interaction between miR-19a-3p and TNF-α was not seen, since the expression of TNF-α was increased in all suicide subjects regardless of psychiatric diagnosis.[22] Results of miR-19a-3p expression from the EVs of the CSF in the present study showed a decrease in miR-19a-3p expression of the suicides which indicates there might be abnormalities of the immune system.

Even though miR-16-5p showed higher expression in suicides, it did not reach statistical significance ($p = 0.0542$). It was connected with depressive disorder in many studies made on humans and animal models, and also showed a contribution to the therapeutic action after antidepressant administration.[52] For example, studies made on depressed patients, and animal models (rats) reported a decrease in miR-16-5p expression in serum,[38] and CSF.[39,53] Contrary, a study made on depressed rat brains (hippocampus) reported an increase in miR-16.[54] Furthermore, with miR-16-5p were associated decreased expression of the BDNF,[54] and increased serotonin level in CSF and raphe.[39,53]

MiR-34c-5p, miR-17-5p, miR-4286, miR-26b-5p, and miR-381-3p did not show statistically significance association with expression from the EVs of the CSF (Table 2). However, several studies made on depressed subjects and suicides showed statistical significant difference for those miRNAs. Mir-34c-5p was tested in two separate studies where it showed an increased expression in subjects with MDD in brain tissue (Brodmann area 44) and blood plasma.[21,40] In another study, higher level of plasma miR-34c-5p was associated with its genetic variation (rs2187473 polymorphism), which could influence immediate and delayed memory performance under disease conditions.[55] MiR-17-5p from neural EVs isolated from blood showed positive correlation with MDD.[41] Its upregulation was detected in blood serum, and two brain regions of the patients with depression.[19,20,42]

Similar results were shown also in the hippocampus of the depressed mouse models.[56] However, one study on blood plasma of MDD subjects did not show any difference miR-17-5p expression.[57] Expression of miR-4286 was studied in two brain regions, Brodmann area 24 of subjects with bipolar disorder died because of suicide, and Brodmann area 10 in suicides. There was a statistical significant difference observed in Brodmann area 24,[23] but not in Brodmann area 10.[45] For the expression of the miR-26b-5p, one study on the peripheral mononuclear cells of the MDD subjects showed its increase,[43] and the second study on the dorsolateral prefrontal cortex of the depressed subjects reported a decrease in miR-26b-5p expression.[44] There was no statistical difference in miR-26b-5p from neural EVs isolated from blood of the MDD subjects.[41]

The limitation of our study is a small sample size (40 samples). This exploratory study should be further validated on a bigger cohort of samples. However, we are following strict inclusion criteria (age between 18 and 65, male sex, hanging as the suicide method) which result in homogenous samples. There is also a substantial difference in age between the suicide and the control group. The age difference comes from the fact that there are more middle-aged men who die due to suicide, while cardiac arrest affects older population. The age difference between the groups might be reduced, if also subjects over the age of 65 would be included in the study. Nevertheless, this could bias our samples with age-related neurodegeneration, which is more pronounced after the age of 65. Limited information from medical records and lack of information about psychiatric condition is another limitation. But, toxicological information from blood and urine that were made during autopsy gave us information about the status at the time of death.

5 | CONCLUSION

In the field of psychiatry, epigenetics might become an important tool for future understanding of brain complexity. Approximately 70% of known miRNAs are expressed in central nervous system where they are involved in regulating neurogenesis and neuroplasticity in developing and adult brain.[16,58,59] Physiological changes contribute to miRNA expression, which is mirrored also in EVs cargo.[23] Several mental disorder studies showed important miRNAs changes and proposed possible (peripheral) biomarkers.[46] MiRNAs changes from brain are reflected in peripheral tissue, therefore studying blood or CSF could carry useful information. Even though blood is easily accessible, CSF holds more information of brain state.[20] Moreover, miRNA profile of one biofluid (for example CSF) is more similar among different subjects than between different biofluids (for example CSF and plasma) within one subject. Therefore, both, blood and CSF should be studied to find overlapping miRNAs among them, which could be used as clinically relevant biomarkers for evaluation of psychiatric condition.[46] Moreover, EVs are, because of their highly enrichment with miRNAs and possibility to pass the blood–brain barrier, gaining interest also in the miRNA research. Improving technique of EVs isolation are enabling enrichment of neural EVs from blood. In clinic, neural EVs from blood would help us monitor state of mental disorder patients in relatively non-invasive way.[59] To conclude, investigating epigenetic changes, specifically miRNAs from CSF, is interesting for understanding suicide and other mental disorders, because of several reasons: miRNAs originate from brain, changes that appear due to psychopathology, or are causing it, can be mirrored in EVs of the CSF, CSF is stable under several freeze thaw and long-term conditions, and possibility for monitoring the drug treatment of mental disorders.[59,60]

FUNDING INFORMATION

This work was funded by the Slovenian Research Agency, grants for Young Researcher, Research Project Z3-2653, and Research Programme P1-0390. Funding source was not involved in research and/or preparation of this article.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available upon reasonable request.

ETHICS STATEMENT

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia.

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How to cite this article: Šalamon Arčan I, Katrašnik M, Kouter K, Zupanc T, Videtič Paska A. Extracellular vesicles from cerebrospinal fluid revealed changes in miR-19a-3p and miR-4516 expression in Slovene male suicides. *Genes, Brain and Behavior*. 2023;22(6):e12868. doi:10.1111/gbb.12868