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The Association of Brain-Related Circulating miRNAs and Delirium Among Cardiac Surgery Patients

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Abstract: Background: Delirium is a neuropsychiatric syndrome that is pathophysiologically related to both mental (dementia, depression) and physical illness. Its occurrence results in a poor prognosis. Specific miRNAs play a role in regulating central nervous system development, neuroinflammation, and neurodegeneration. This study investigates whether specific miRNAs (miR-9-3p, miR-34c-5p, miR-96-5p, miR-183-5p, and miR-374-3p) related to brain function are associated with an increased risk of postoperative delirium. **Methods:** A total of 224 adult individuals scheduled for elective cardiac surgery were eligible to participate in the study. Prior to surgery, each patient underwent psychiatric evaluation to identify major depressive disorder based on the DSM-5 criteria. Delirium diagnosis was established with the use of the Confusion Assessment Method. Following miRNA expression profiling, cDNA synthesis was conducted on 60 delirium patients and 60 randomly selected non-delirium individuals. MiRNA dqPCR analysis was performed on the serum samples obtained one day before and the day after surgery. **Results:** Of the 177 patients finally included, 34% (61 cases) experienced delirium. Univariate comparisons revealed that preoperative miR-96-5p ($p=0.05$) and miR-183-5p ($p=0.001$), along with postoperative miR-34c-5p ($p=0.009$), miR-96-5p ($p=0.07$), and miR-183-5p ($p=0.05$), were associated with the risk of post-surgery delirium. However, after conducting multivariate logistic regression analysis, only miR-183-5p was found to be independently associated with the risk of delirium development. Other predictors of delirium included an ongoing episode of depression, peripheral vascular disease, female gender, active smoking, and increased postoperative pCO2 concentration. **Conclusion:** The current study revealed that preoperatively decreased expression of miR-183-5p predicts delirium development after cardiac surgery.

Keywords: miR-183-5p; delirium; oxidative stress; neuroinflammation; antioxidant activity

1. Introduction

Delirium is a neuropsychiatric syndrome that is commonly observed after major surgery, particularly among elderly patients with physical and psychiatric comorbidities, as well as those with impaired cognitive status [1,2]. It is characterized by sudden onset, fluctuations in attention and cognitive deficits, reduced awareness, and altered psychomotor activity. The frequency of delirium in elderly hospitalized individuals is exceptionally high, with rates of up to 70% [3]. Similarly, patients hospitalized in intensive care units due to COVID-19 infection have comparable rates of

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delirium at 73% [4]. Unfortunately, delirium is associated with poor prognosis, increased mortality, and impaired functioning even after the syndrome resolves [5].

The risk factors for delirium are well-known and described. However, investigations into its pathophysiology have only recently begun. The processes underlying delirium include an imbalance in the hypothalamic-pituitary-adrenal (HPA) axis, increased oxidative stress, and inflammation [6,7]. Despite significant progress, the role of miRNA in the development of delirium remains unclear.

MicroRNAs (miRNAs) are small, single-stranded RNA molecules that play a crucial role in post-transcriptional gene expression. Their post-transcriptional activity includes both the inducement of mRNA degradation and the stimulation of gene expression [8,9]. Specific miRNAs are recognized as regulators of three basic areas: central nervous system (CNS) development, neuroinflammation, and neurodegeneration.

The role of miRNAs in neural development was first demonstrated through conditional knockout mice experiments involving enzymes responsible for miRNA biogenesis [9]. For instance, miR-9 is highly expressed in neural precursors and regulates the number of neural stem cells [10,11]. Its overexpression controls proliferation and promotes neural differentiation by suppressing the orphan receptor [12].

Fu et al. (2020) conducted a study that demonstrated that miR-34c-5p is significantly reduced in individuals with drug-resistant epilepsy as compared to the control group [13]. The findings of the study suggest that the decrease in miR-34c-5p levels in drug-resistant epilepsy is responsible for inducing neuroinflammation, leading to the loss of hippocampal neurons and worsening of the disease. Additionally, Tu and Hu's (2021) research found that the lowered expression of miR-34c-5p is associated with cerebral ischemia/reperfusion injury in experimental artery occlusion ischemic models and reperfusion-induced models in animal subjects [14]. The authors suggest that inflammatory and apoptotic signaling pathways may mediate the above process.

A recent study revealed that the upregulation of miR-96-5p increases the level of the glutamate transport-associated protein (GTRAP3-18), leading to a decrease in the concentration of the excitatory amino acid transporter (EAAC1). EAAC1 regulates the concentration of neuronal glutathione (GSH), an important neuroprotective antioxidant. When the level of EAAC1 decreases, GSH activity also diminishes, resulting in oxidative stress, neuronal damage, and the onset of neurodegenerative processes [15].

Although some miRNAs have been identified as regulators of the CNS and mediators of neuroinflammation, only a few are critical for regulating synaptic plasticity and cognitive status. One of these miRNAs is miR-9-3p, which is highly expressed in developing and mature brains [16]. Both miR-9-3p and miR-9-5p are produced from the miR-9 precursor and are involved in developing and losing neurons, synapses, and microglia activation [16,17]. The level of miR-9-3p is decreased in neurodegenerative diseases such as Huntington's and Alzheimer's disease (AD) [18,19]. It has been described as a biomarker differentiating between Parkinson’s disease (PD) and Multiple System Atrophy (MSA) [20].

Das Gupta et al. (2021) found that after experimental traumatic brain injury (TBI), plasma miR-9-3p levels increase [21]. The study showed that in patients with severe TBI, plasma miR-9-3p levels were 6.5- and 9.2-fold higher compared to those with mild TBI and the control group, respectively. These findings illuminate miRNA's role in neuronal loss and brain tissue repair. In the study conducted by Wang et al. (2020), it was found that miRNA-183-5p expression in the mouse brain decreased after experimental intracerebral hemorrhage (ICH) [22]. The authors investigated the effect of miRNA-183-5p on the injury and repair of brain tissue after ICH by injecting miRNA-183-5p agomir or miRNA-183-5p antagomir into the lateral ventricles of mice with experimentally induced ICH. After several days of treatment, mice treated with exogenous miRNA-183-5p showed less brain edema, neurobehavioral defects, inflammation, oxidative stress, and ferrous deposition than antagomir-treated mice. Two studies have identified the specific miRNA involved in the development of postoperative delirium [23,24]. The first study found a correlation between microRNA-320 and postoperative delirium in patients undergoing tibial fracture internal fixation

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surgery [23]. The second study revealed that preoperative miR-210 could potentially predict the occurrence of postoperative delirium in elderly gastric cancer patients undergoing curative resection [24]. Recent research has shown that certain miRNAs can target genes that regulate the permeability of the blood-brain barrier (BBB) in both animal and human in vitro models. This discovery suggests that miRNAs have a role in modifying the integrity of the BBB. For instance, Coxsackievirus A16 can penetrate the BBB and decrease the expression of miR-1303 [25]. As a result, junctional complexes are disrupted by directly regulating matrix metallopeptidase 9 (MMP9), ultimately leading to pathological changes in the CNS [25]. The purpose of the present study was to investigate whether specific miRNAs (miR-9-3p, miR-34c-5p, miR-96-5p, miR-183-5p, and miR-374-3p) related to brain function are associated with an increased risk of delirium among patients who have undergone cardiac surgery.

2. Results

Elective cardiac surgery was performed in two hundred ninety-four patients during the study period; of these, 58 subjects did not meet the inclusion criteria since they underwent different than CABG/CABG plus CVR surgery (isolated CVR surgery without CABG, including minimally invasive mitral valve repair [n = 58]), and 12 individuals did not sign an informed consent). Of the 224 patients who signed their informed consent and were enrolled, four patients were lost to follow-up since they died before the observational period was completed, and 43 individuals had incomplete study data (these patients were not included in the analysis due to failure during samples collection or inappropriate samples collection (coagulation), n = 28; and due to incomplete postoperative delirium evaluation, n = 15). The incidence of delirium among the 177 remaining patients was 34% (61 delirium cases). After the miRNA expression profiling, cDNA synthesis was performed in 60 delirium patients and 60 randomly selected non-delirium individuals (simple randomization using the computer random number generation program). miRNA dqPCR analysis was performed in the serum samples obtained one day before and the day after surgery (see the Laboratory measurements section).

2.1. Univariate and Multivariate Comparisons

The median preoperative and postoperative miRNA expression levels and oxidative stress biomarker concentrations in the whole population are presented in Table 1.

Table 1. The median miRNA expression and biomarker concentrations in the whole study population.

miRNA	Pre-operative level copies/ml[a]	Post-operative level copies/ml[a]
miR-9-3p	79 (13.0 - 172.1)	101.4 (20.7- 191.9)
miR-34c-5p	12.5 (0.0 - 45.4)	25.7 (0 -108.1)
miR-96-5p	311.6 (110.2 -696.8)	156.7 (68.7 – 325.7)
miR-183-5p	78.9 (14.3 – 179.2)	73.4 (10..4 – 168.2)
miR-374-3p	2.8 (0.0 -30.04)	0 (0.0 – 16.3)
Superoxidase dismutase (ng/ml)	2.68 (2,06-3.53)	2.13 (1.62-3.01)
Antioxidant activity (µmol/l)	2.1 (1.3–2.9)	1.8 (1.2–2.6)

[a]For continuous variables, the medians and interquartile ranges (IQRs) are given.

The findings of the univariate analysis of demographics, comorbidities, and factors related to anesthesia and surgical procedures are shown in Tables 2–4.

Table 2. The expression of preoperative and postoperative miRNA in univariate comparisons.

Variable	Non-delirious[a]	Delirious[a]	Effect size[b]	P value[c]
Preoperative miR-9-3p	87.4 (34.7-187.2)	53.8 (5.1-156.6)	0.32	0.39
Preoperative miR-34c-5p	20.5 (0.0-63.1)	8.0 (0.0-42.5)	0.19	0.28

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Preoperative miR-96-5p	368.2 (169.7-832.5)	165.7 (55.0-507.9)	0.49	0.05
Preoperative miR-183-5p	210.5 (67.7-347.6)	53.02 (9.6-173.2)	0.77	**0.0005**
Preoperative miR-374-3p	9.3 (0.0-39.7)	0 (0.0-11.2)	0.34	0.26
Postoperative miR-9-3p	118.5 (45.04-185.9)	57.13 (0-201.9)	0.32	0.39
Postoperative miR-34c-5p	51.4 (15.7-121.4)	7.6 (0-69.6)	0.56	**0.009**
Postoperative miR-96-5p	187.2 (96.5-364.2)	119.2 (33-278,6)	0.47	0.07
Postoperative miR-183-5p	89.1 (14.5-247.)	39 (0-98.1)	0.49	0.05
Postoperative miR-374-3p	0 (0-18.0)	0 (0-15.4)	0.09	0.99

[a] For continuous variables, the medians and interquartile ranges (IQRs) are given. [b] For continuous variables, Cohen's d coefficient was calculated; for categorical variables, Cramer's V coefficient was presented. [c] P value with Sidak correction for multiple comparisons was calculated. Significant values are in [**bold**].

Table 3. Perioperative characteristics in univariate comparisons.

Variable	Non-delirious[a]	Delirious[a]	Effect size[b]	P value[c]
CABG plus valve surgery	2 (3.3%)	8 (23.3%)	0.18	**0.04**
Duration of surgery (h)	4 (3 – 4.5)	4 (3.4 – 4.4)	0.15	0.41
Extracorporeal circulation	40 (66.7%)	51 (85%)	0.21	**0.02**
Intraoperative circulatory support	16 (26.7%)	17 (28.3%)	0.24	0.16
Post-op. hyperthermia >38°C	6 (10%)	9 (15%)	0.08	0.40
Post-op. pO2 ≤60 mmHg	5 (8.3%)	11(18.3%)	0.15	0.10
Post-op. pCO2 ≥45 mmHg	7 (11.7%)	18 (30%)	0.40	**0.01**
Plasma transfusion > 1 unit	6 (10%)	9 (15%)	0.08	0.40
Blood transfusion > 4 units	1 (6.7%)	5 (8.3%)	0.15	0.21

CABG: Coronary Artery Bypass Graft Surgery; pO2: partial pressure of oxygen; pCO2: partial pressure of carbon dioxide. [a]For continuous variables, the medians and interquartile ranges (IQRs) are given; for categorical variables, the number of observations (n) and fraction (%) were calculated. [b] For continuous variables, Cohen's d coefficient was calculated; for categorical variables, Cramer's V coefficient was presented. [c] P value with Sidak correction for multiple comparisons was calculated. Significant values are in [**bold**].

Univariate comparisons revealed that preoperative miR-96-5p (p=0.05) and miR-183-5p (p=0.001), and postoperative miR-34c-5p (p=0.009), miR-96-5p (p=0.07), and miR-183-5p (p=0.05) were associated with the risk of post-surgery delirium. However, according to the results of multivariate logistic regression analysis, only preoperative miR-183-5p was independently associated with the risk of postoperative delirium development (Table 4). Other predictors of post-surgery delirium included an ongoing episode of depression, peripheral vascular disease, female gender, active smoking, and an increased postoperative pCO2 concentration.

Table 4. Factors independently associated with delirium after cardiac surgery revealed in a multivariate stepwise logistic regression analysis.

Variable	Coefficient	Standard Error	OR (95% CI)	P value
Depression	2.53	0.79	12.6 (2.7-59.2)	< 0.001
Preoperative miR-183-5p	-0.002	0.001	0.99 (0.995-0.999)	0.005
Postoperative pCO2 >= 45	1.32	0.62	3.7 (1.1-12.6)	0.03
Cigarette smoking	0.91	0.46	2.4 (1.007-6.15)	0.05
Gender Female	1.26	0.56	3.5 (1.2-10.5)	0.02
Peripheral vascular disease	1.38	0.68	3.9 (1.04-15.2)	0.04
Constant	-1.005	0.40	-	0.01

pCO2: partial pressure of carbon dioxide. The regression model is statistically significant: $\chi 2=49.641$, df=6, p <000.1; Hosmer–Lemeshow test: $\chi 2=11.203$, p=0.190; Nagelkerke $R^2=0.451$.

2.2. Optimal miRNA Thresholds and Correlations with Other Analyzed Variables

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According to the ROC analysis, the most optimal cutoff value of preoperative miR-183-5p that predicts the development of delirium was ≤ 193 copies/ml, with a sensitivity of 82% and specificity of 57%, a positive predictive value of 0.65 and negative predictive value of 0.75 (area under the curve = 0.7; standard error = 0.05; 95%CI: 0.61 to 0.80; p<0.001).

It is that in the group of patients who did not experience delirium, there was a trend towards a significant correlation between preoperative miR-183-5p expression and preoperative superoxidase dismutase (SOD) activity (Spearman's rank correlation 0.264; p=0.09). However, a significant positive correlation was found between preoperative SOD activity and the preoperative levels of miR-96-5p ($p < 0.05$). Additionally, there was a significant positive correlation between postoperative miR-96-5p and preoperative plasma antioxidant activity, which is a marker that reflects the total activity of all plasma antioxidative agents (Spearman's rank correlation 0.199; p=0.04).

3. Discussion

The current study revealed that preoperatively decreased expression of miR-183-5p predicts delirium development after cardiac surgery.

In previous studies, upregulation of miR-183-5p has been reported to alleviate liver and brain injury induced by ischemia-reperfusion (I/R) [26]. Moreover, exosomal miR-183-5p was revealed to protect against myocardial I/R injury by targeting forkhead box protein O1 (FOXO1), reducing apoptosis and oxidative stress in I/R cardiomyocytes and improving cardiac function [27]. Zhu et al. study (2020) evaluated the effects of miR-183-5p on ischemia injury using ischemic models of mouse brains exposed to transient middle cerebral artery occlusion and Neuro-2A (N2A) neuroblastoma cells exposed to oxygen-glucose-deprivation (OGD) [26]. Their study investigated ischemia, miR-183-5p expression, N2A cells viability, and apoptosis-associated proteins' expression. The results revealed that miR-183-5p expression was decreased, and brain damage was increased in ischemic mice compared with the sham group. Furthermore, N2A cells exposed to ischemia were characterized by lower miR-183-5p expression levels and increased apoptosis compared with the control group. Following the administration of agomiR-183-5p, cerebral ischemic injury and apoptosis were reduced in the stroke model and OGD-induced N2A cells.

Also, Wang et al. (2020) conducted a study to investigate the effect of miR-183-5p on brain injury in animal models [22]. Their analysis revealed that the expression of miR-183-5p decreased in the mouse brain after experimental intracerebral hemorrhage (ICH). However, mice treated with exogenous miRNA-183-5p showed improved brain edema, neurobehavioral defects, inflammation, oxidative stress, and ferrous deposition three days after ICH onset compared to control non-treated mice. As one of the miR-183-5p targets is heme oxygenase-1 (HO-1) (a molecule reported to exacerbate ICH brain injury), the authors investigated the association between agomir-183-5p and antagomir-183-5p injections, HO-1 levels, and cerebral injury. In the agomir group, the expression of HO-1 decreased significantly ($P < 0.05$), whereas there was no difference in its expression between the antagomir group and the ICH group. The study revealed that the positive impact of miR-183-5p on brain injury was related to inflammation and oxidative damage reduction via HO-1 inhibition.

Roser et al. investigated the role of microRNAs in Parkinson's disease, hypothesizing that glial cell line-derived neurotrophic factor (GDNF) may enhance the survival of dopaminergic (DA) neurons in Parkinson's disease (PD) models [28]. They demonstrated that transfecting synthetic miR-182-5p and miR-183-5p resulted in increased neurite outgrowth and provided neuroprotection for DA neurons both in vitro and in vivo, similar to the effects of GDNF. This effect was associated with decreased expression of the transcription factors FOXO3 and FOXO1 and enhanced PI3K-Akt signaling. Both of these transcription factors are known to promote neuronal apoptosis in response to oxidative stress and affect neurite growth [29].

In another study of animal models, extracellular vesicles derived from bone marrow mesenchymal stem cells carrying miR-183-5p were used to assess the impact on a diabetic intracerebral hemorrhage. The study showed that miR-183-5p alleviated neuroinflammation and oxidative stress via the PDCD4/NLRP3 pathway [30].

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Interestingly, Zhou et al. revealed that extracellular vesicle-encapsulated miR-183-5p has a protective effect against the methamphetamine-induced dependence model in mouse brains by targeting neuregulin 1 [31]. Furthermore, in the prospective cohort study conducted among concussed children with and without persistent post-concussive symptoms (PPCS), expression of miR-183-5p was evaluated over time post-concussion. The results indicated statistically significant miRNA 183-5p overexpression after concussion in children with PPCS compared to children without PPCS [32].

The present study is the first to reveal the role of miR-183-5p in the development of neurocognitive disorders. Specifically, the analysis showed that decreased miR-183-5p expression independently predisposes to postoperative delirium development among CVD patients. Available studies suggest that the mechanisms responsible for the impact of miR-183-5p on brain injury involve neuroinflammation and oxidative stress. As cardiac surgery burdens the brain's functioning, lower expression of the miR-183-5p may contribute to postoperative delirium via less efficient inhibition of neuroinflammatory and oxidative stress processes. Our previous studies revealed that CABG patients with decreased preoperative antioxidant activity (less efficient antioxidative mechanisms) and those with depressive episodes complicated with lower postoperative antioxidant activity are at significantly higher risk of delirium after cardiac surgery [6]. As pre- and postoperative antioxidant capacity levels were negatively correlated with postoperative soluble receptor for advanced glycation end products (sRAGE) concentration, we concluded that sRAGE overexpression may be a protective mechanism against increased oxidative stress and subsequent cell damage. In another study, we found that individuals with less efficient baseline antioxidative mechanisms have a higher postoperative peak of myeloperoxidase (MPO), a lysosomal enzyme known for its strong pro-oxidative and pro-inflammatory properties [2]. Consequently, these patients were more susceptible to experiencing delirium after surgery. Additionally, a higher preoperative plasma concentration of monocyte chemoattractant protein-1 (MCP-1) - a key chemokine involved in neuroinflammation and myelin degradation - has been identified as a predictor of postoperative delirium development [7]. Other studies among non-cardiac surgery patients also revealed the role of inflammation in postoperative delirium development [33,34].

The reason for decreased preoperative miR-183-5p expression in the studied population is unknown. However, present analysis revealed that individuals with higher preoperative superoxide dismutase (SOD) concentration who did not develop delirium were characterized with increased miR-183-5p levels (a trend towards significance; $p<0.1$). The same positive correlation was observed between preoperative SOD activity and miR-96-5p levels ($p<0.05$). Additionally, a significant positive correlation between postoperative miR-96-5p and preoperative plasma antioxidant capacity was revealed ($p<0.05$).

SOD is the first line of antioxidative defense, and its increased activity was associated with protective effects against mortality from cancer [35] and with lower all-cause mortality in older women [36]. Antioxidant capacity (AC) reflects the cumulative action of all antioxidants present in plasma, and its lower measures may indicate higher vulnerability to diseases related to oxidative stress.

Interestingly, in previous studies, increased miR-183-5p expression was significantly upregulated post-concussion in children with persistent post-concussive symptoms (PPCS) as compared to those without PPCS. The different distributions of the identified miRNAs in children with vs. without PPCS may signal a differential physiological response to the concussive injury or its subsequent repair [32].

Based on the aforementioned findings, it can be hypothesized that specific miRNAs may have protective mechanisms against excessive oxidative stress and neuronal damage. The presence of miR-183-5p may suggest curative activity, while decreased levels of this miRNA may increase the risk of neuronal damage after cardiac surgery and other traumas.

Limitations

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This well-designed, innovative study does have some limitations. MiRNA was measured only once before the operation and once after; there were no additional tests to evaluate how miRNA expression changed over time postoperatively. Additionally, the entire study group consisted of patients with advanced cardiovascular disease, and there was no control group without CVD for comparison. Recent studies have shown that patients with carotid atherosclerosis exhibit higher serum levels of miR-183-5p than healthy individuals [37,38]. Increased levels of circulating miR-183-5p were also detected in patients with acute coronary syndrome (ACS) and non-ST-segment elevation myocardial infarction (NSTEMI) [39,40], with the observation that serum miR-183-5p levels positively correlated with the Gensini score and hs-CRP [35]. Also, further investigations indicated a positive correlation between circulating miR-183-5p levels and coronary artery disease (CAD) compared to non-CAD individuals [41]. According to the studies previously mentioned, miR-183-5p has a protective effect not only on neurons but also on cardiomyocytes. Our patient group consisted of individuals with CVD, making it challenging to determine whether the higher expression of miR-183-5p in those with a lower risk of delirium was linked to a cardioprotective or neuroprotective mechanism or a general effect against oxidative stress. This raises the question of whether the direct examination of miR-183-5p overexpression in neurons and cardiomyocytes should be pursued.

4. Materials and Methods

The study was approved by the Ethics Committee of the Medical University of Lodz, Poland; Approval Number RNN/95/17/KE. The procedures used in the study were under the ethical standards of the Declaration of Helsinki. Adult individuals scheduled for elective cardiac surgery between April 2017 and November 2019 were eligible to participate in the study. The patients were included if they signed informed consent and were presented for isolated CABG surgery or CABG surgery with cardiac valve replacement (CVR). Patients with CABG underwent both on-pump (without cardiopulmonary bypass (CPB)) and off-pump (with CPB) surgery; however, the impact of CPB on the risk of postoperative delirium was controlled in the statistical analysis. The exclusion criteria were as follows: concomitant surgery other than CABG or CABG with CVR; preoperative delirium; active alcohol or other substance addiction (abstinence period shorter than three months); illiteracy; pronounced hearing and/or visual impairment. The participants were recruited consecutively.

Neuropsychiatric Assessment

The day before the scheduled operation, patients' cognitive status was assessed using the Mini-Mental State Examination (MMSE) [42]. The criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) were utilized to diagnose Major Depressive Disorder (MDD) and anxiety disorders [43]. To screen for delirium, the Confusion Assessment Method for the Intensive Care Unit (CAM-ICU) and the Memorial Delirium Assessment Scale (MDAS) were employed, with a cut-off score of 10 [44,45]. The treatment team received training on recognizing delirium symptoms and was instructed to notify clinicians if they observed any changes in patients' cognition, consciousness, or behavior. Each patient underwent delirium screening once daily for the first five days following surgery, and additional assessments were conducted if the treatment team reported any changes in the patient's mental or behavioral condition.

Anesthesia

For induction of anesthesia, fentanyl 5- 10 mcg/kg, propofol 1-2.5mg/kg, and rocuronium 0.6-1.0mg/kg were used. During the maintenance phase, fentanyl in continuous intravenous infusion in doses of 2- 10 mcg/kg/h, propofol 3-10mg/kg/h, and interrupted doses of rocuronium were administered. Ventilation was provided with a breathing mixture of FiO2 0.5 and air to maintain end-tidal CO2 at 35- 45 mmHg. From surgical incision to cardiopulmonary bypass connection, sevoflurane 0.5-2 vol% was used. After surgical intervention, the patients were transferred to the ICU,

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where mechanical ventilation was continued. Until extubation, morphine in continuous infusion of 1-2mg per hour and propofol perfusion at a rate of 1-2mg/kg/h were used for sedation. The criteria for extubation were as follows: arterial blood gases and oxygen saturation>92% and stabilization of hemodynamic parameters.

Surgery

Patients who underwent CABG or CABG with concomitant valve surgery were operated on through median sternotomy and on cardiopulmonary bypass (CPB) under normothermia. The anterograde DelNido cardioplegia was used in all patients during the operation. In some cases, patients who underwent CABG were operated on without CPB (off-pump CABG), on a beating heart, either through median sternotomy or through left-sided mini-thoracotomy.

Laboratory Measurements

Venous blood samples were taken twice during the study period: the day before the surgery (baseline measurement) and on the first postoperative day, between 07:00 and 09:00 a.m. The blood samples were centrifuged at 7,000 rpm for 10 minutes and frozen at -80°C until biochemical parameters were determined.

Exosome Isolation

According to the manufacturer's instructions, total exosomes were extracted using the miRCURY Exosome Serum/Plasma Kit (Cat No. 76603; Qiagen). In brief, plasma was thawed on ice and centrifuged at 3000 × g for 10 min at 4°C. 800 µL of supernatant was transferred to a new tube, and 8 µL of thrombin (stock concentration of 500 U/mL) was added and incubated for 5 min at room temperature. The plasma was centrifuged at 10,000 x g for 5 min, and 700 µL of the supernatant was transferred to a new tube. Then 280 µL Precipitation Buffer A was added, incubated for 60 min at 4°C, and centrifuged twice at 500 x g for 5 min to remove the supernatant. The pellet was resuspended in 240 µL of Resuspension Buffer for RNA extraction.

Isolation and Purification of miRNA

Total RNA, including miRNA, was extracted using the miRNeasy Mini Kit (Cat No. 217004; Qiagen) according to the manufacturer's protocol with one minor variation. After the sample was mixed with 700 µL of QIAzol Lysis Reagent, 5 µL of 5 nM cel-miR-39-3p (Cat No. 4464066, Ambion) was added as an exogenous spike-in control. RNA was eluted from spin columns in 30 µL of RNase-free water and stored at -80°C until use.

Expression Profile of miRNA Genes

A broad miRNA profiling was carried out using TaqMan® Human MicroRNA Array A and B (Cat No. 4444913, Thermo Scientific, Waltham, MA, USA) as described by the manufacturer. Briefly, three µL of RNA extracts were reverse-transcribed using MegaplexTM RT Primers Human Pool A and B (Cat No. 4444745, Thermo Scientific) in a final volume of 7.5 µL. cDNA targets were then pre-amplified using 2.5 µL of the RT product in a 25 µL pre-amplification reaction. The PCR mixture for each array contained 450 µL of TaqMan Universal PCR Master Mix, nine µL of diluted PreAmp product, and 441 µL of nuclease-free water. The plates were inoculated with 100 µl of the mixture into each port and sealed. Amplifications were performed using the 7900HT Fast Real-Time PCR System (Thermo Scientific) under the following conditions: 2 min at 50°C, 10 min at 94.5°C, and 40 cycles each for 30 s at 97°C and 1 min at 57°C. The expression levels of 754 human miRNA genes were assessed in serum samples of 4 patient groups: samples obtained before surgery from patients who developed delirium (n=10), samples obtained after surgery from patients who developed delirium (n=10), samples obtained before surgery from non-delirious patients (n=10), samples obtained after

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surgery from non-delirious patients (n=10). Only miRNAs with Cq < 35 were considered as detected. miRNAs with altered expression profiles were chosen for further investigations.

cDNA Synthesis and Digital Quantitative PCR

According to the manufacturer's instructions, the purified total RNA was reversely transcribed into cDNA using the TaqManTM advanced miRNA cDNA Synthesis Kit (A28007, Thermo Scientific, Waltham, MA, USA). The cDNA synthesis protocol consisted of a poly(A) tailing reaction, a ligation reaction adding an adaptor sequence, and a reverse transcription reaction. 5µL of RT product was pre-amplified to increase the amount of cDNA for all miRNAs (miR-Amp reaction) uniformly and stored at -20°C. Selected miRNAs were profiled using digital quantitative PCR with a QX200 droplet digital PCR system (Bio-Rad) according to the manufacturer's instructions. The ddPCR mixture was composed of 11 µL of 2 × ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 1.1 µL of appropriate TaqMan Advanced miRNA Assay: hsa-miR-96-5p (Assay ID: 478215_mir), hsa-miR-34c-5p (Assay ID: 478052_mir), hsa-miR-9-3p (Assay ID: 4782151_mir), hsa-miR-183-5p (Assay ID: 477937_mir) or hsa-miR-374b-3p (Assay ID: 479421_mir), 8.9 µL DNase/RNase free MilliQ water and 1 µL of 10 time-diluted cDNA, in a final reaction volume of 22 µL. Bio-Rad QX200 droplet generator was used to partition each PCR reaction into up to 20,000 nano-sized droplets by loading 20 µL of the reaction mixture and 70 µL of droplet generation oil for probes (Bio-Rad) onto matched wells of a DG8 cartridge (Bio-Rad). 40 µL of the droplet/oil mixture was transferred to a 96-well plate (Bio-Rad). The plate was then heat-sealed using a PX1 PCR plate sealer (Bio-Rad) set to run at 180˚C for 5 s. The PCR was performed in a T100 Thermal Cycler (Bio-Rad). The PCR reaction setups and thermal cycling conditions used for individual miRNA quantification are summarized in Appendix A, Table A1.

The fluorescence signals were measured by the QX200 Droplet Reader (Bio-Rad). The positive droplets containing amplified products were distinguished from negative droplets by applying a threshold above the negative droplets. Reactions with more than 10,000 accepted droplets per well were analyzed using QuantaSoft™ Analysis Pro software version 1.0.596 (Bio-Rad). Subsequently, the results were converted into copies/1 mL of the input material concerning the input plasma volume and dilutions at the RT and PCR reaction levels.

Statistical Analysis

Quantitative variables are expressed as medians and interquartile ranges (IQRs). For categorical variables, the number of observations (n) and fraction (%) were calculated. Normality was tested using Shapiro-Wilk's test for normality. Differences between two independent samples for continuous data were analyzed using the Mann-Whitney U test (since the distributions of variables were different from normal). The effect size for continuous variables was calculated with Cohen's d. For categorical variables, statistical analysis was based on the chi-squared test or Fisher's exact test. Cramer's V coefficient was calculated to assess the effect size for categorical variables. Spearman's rank correlation coefficients were calculated to determine the correlation between two quantitative variables. The minimum study sample size was calculated using the power analysis, estimating the expected effects from our previous studies and assuming an alpha level of 0.10 and a power of 80% (the minimum sample size for each group is 37 patients). To evaluate the discriminant power of miRNAs, receiver operating characteristic (ROC) curves were drawn (Area Under Curve with Standard Error was given), and optimal decision thresholds (based on Youden's index value) were found. The sensitivity, specificity, and positive and negative predictive values were calculated. Odds ratios with 95% confidence intervals were also presented. All investigated miRNAs and other factors significant in univariate comparisons ($p < 0.10$) were included in a forward stepwise logistic regression model to identify independent risk factors for delirium. The results were considered significant for $p < 0.05$. The calculations were performed using STATISTICA (version 13.3, 2017; StatSoft, Inc., Tulsa, OK, USA) and the R-project (the rcompanion package).

5. Conclusions

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The current study indicates that patients with reduced preoperative expression of miR-183-5p face a greater risk of experiencing postoperative delirium. This association may be related to increased oxidative stress and neuroinflammation in this patient group.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Lodz, Poland (RNN/95/17/KE 14.03.2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.
Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. This article is a revised and expanded version of a conference poster: "Decreased preoperative miR 183-5p expression and an episode of depression are the independent predictors of delirium after cardiac surgery", which was presented at the 35th ECNP Congress, 15-18 October 2022; Vienna, Austria [46].

Appendix A

Table A1. The PCR thermal cycling conditions (T100 Thermal Cycler, BioRad).

	Cycling step	Temperature, °C	Time	Ramp rate	No of cycles
miR_96-5p	Enzyme activation	95	10 min	1°C/sec	1
	Enzyme activation	94	30 sec		50
	Annealing/extension	62.5	1 min		50
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_34c-5p	Enzyme activation	95	10 min	2.5°C/sec	1
	Denaturation	94	30 sec		40
	Annealing/extension	60	1 min		40
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_9-3p	Enzyme activation	95	10 min	1°C/sec	1
	Denaturation	94	30 sec		50
	Annealing/extension	62.5	1 min		50
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_18	Enzyme activation	95	10 min	1°C/sec	1
	Denaturation	94	30 sec		50
	Annealing/extension	62	1 min		50

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	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_374-3p	Enzyme activation	95	10 min	2.5°C/sec	1
	Denaturation	94	30 sec		40
	Annealing/extension	60	1 min		40
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1

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