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Evaluation of Circulating MicroRNAs in Schizophrenia: From Epigenomic Dysregulation to Potential Biomarkers

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Abstract: To evaluate the expression profile of circulating miRNAs in patients with schizophrenia (hsa-miR-34a, miR-449a, miR-564, miR-432, miR-548d, miR-572, and miR-652) in relation to individual negative controls for the disease. This was an analytical, case-controlled, cross-sectional study, using samples previously collected from patients diagnosed with schizophrenia (N = 650) and a control group (N = 924). Samples were analyzed after RNA extraction and quantification. After making a general comparison between the case and control groups, regardless of gender and other variables, all seven miRNAs showed statistically significant differences (p-value < 0.05). This also occurred in the variables gender, smoking, and alcoholism. Thus, the results indicated that depending on the clinical characteristics in the face of suspected schizophrenia, the miRNAs explored here seem to work as possible biomarkers, as they demonstrated, at various times, important differences between the studied groups.

Keywords: schizophrenia; microRNA; mental health; biomarkers

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1. Introduction

Schizophrenia is a severe and intricate pathology categorized within the spectrum of psychotic mental disorders, impacting approximately 0.5–1% of the global population. This disorder is characterized by a chronic nature and is recognized as one of the top 15 leading causes of disability [1]. Schizophrenia is classified as a psychotic disorder, situated within the broader context of psychosis, and is identified as a syndrome comprising delusions, hallucinations, and cognitive disturbances, resulting in a progressive deterioration of reality contact [2]. The mortality rate associated with schizophrenia is significantly elevated, approximately 2.58 times higher than that of the general population, with a noted increase in rates since the 1970s [3]. Additionally, individuals diagnosed with schizophrenia often experience low rates of clinical improvement, social integration [4], and a marked reduction in life expectancy [5].

Evidence indicates that the incidence of schizophrenia is higher among individuals with a familial history of the disorder, with incidence rates reaching 35% higher in offspring of two affected parents [6]. Within this framework, limited research has thoroughly examined the nature of this disparity with respect to ethnicity. In the majority of studies, ethnicity has been regarded as a confounding variable, or the sample sizes have been insufficient for stratified analysis. Studies have shown that, irrespective of ethnicity, people with severe mental illness experience excess mortality, highlighting an urgent need to address preventable causes within this population. Interestingly, research has also indicated a

reduced mortality risk in black African, black Caribbean, and South Asian groups with severe mental illness when compared to a white British reference group with the same condition. This disparity may be attributed to several factors, including socioenvironmental differences, variations in underlying physical health, and differences in the prescribing of psychotropic medications. Understanding these factors could be crucial for improving mortality outcomes in all individuals with severe mental illness. This observation is critical given that many risk factors for mortality associated with severe mental illnesses, such as cardiovascular disease and diabetes, are documented to be more prevalent among certain ethnic minority groups in comparison to white British, European, and non-Hispanic white American populations [7].

Regarding the clinical diagnosis of schizophrenia, specific clinical criteria must be assessed, encompassing both positive and negative symptoms [8]. The etiology and triggers of the disorder encompass a genetic predisposition, with clinical manifestation occurring subsequent to exposure to environmental stimuli [9]. Substantial evidence suggests that these environmental factors can influence epigenetic mechanisms that regulate gene expression [10]. To enhance understanding and facilitate the clinical diagnosis of schizophrenia, established criteria include the evaluation of positive and negative symptoms. Positive symptoms comprise (1) delusions, (2) hallucinations (particularly auditory), (3) disorganized speech, and (4) disorganized and/or catatonic behavior. Negative symptoms primarily involve (5) a profound lack of motivation and diminished emotional expression, frequently leading to compromised social interaction. Cognitive impairments, often manifested as deficits in concentration, working memory, and learning capabilities, are also commonly observed. From an etiological perspective, schizophrenia is widely classified into subtypes including paranoid, disorganized, catatonic, undifferentiated, and residual schizophrenia [8].

One of the primary avenues for future therapeutic interventions lies in the utilization of epigenetic pharmacotherapy, focusing on specific biomarkers, commonly referred to as targets, and preferably integrated with biopsychosocial strategies [11]. Among the potential biomarkers, microRNAs (miRNAs) present as stable and reliable indicators following extensive studies utilizing peripheral tissue samples [12]. MicroRNAs are small non-coding RNA molecules that modulate gene expression at the post-transcriptional level [11]. Certain miRNAs have been previously identified as promising biomarkers for schizophrenia in peripheral samples, including hsa-miR-34a, miR-449a, miR-564, miR-432, miR-548d, miR-572, and miR-652 [13,14]. Notably, hsa-miR-34A has been frequently identified in peripheral blood samples, further substantiated by its documented presence in the dorsolateral prefrontal cortex (Brodmann area 46) of individuals diagnosed with schizophrenia [15], as well as its presence in plasma [16,17].

Consequently, the objective of this study was to evaluate the profile of epigenetic parameters in patients with schizophrenia through an analysis of specific circulating miRNAs (hsa-miR-34a, miR-449a, miR-564, miR-432, miR-548d, miR-572, and miR-652).

2. Materials and Methods

2.1. Study Design

This is an analytical, case-controlled, cross-sectional study, in which patients diagnosed with schizophrenia (N = 650) were studied, 300 female patients and 350 male patients, from a selected sample at the Hospital of Clínicas Gaspar Vianna (Belém, PA, Brazil) from July 2005 to June 2015. The clinical presentation was collected and recorded and the diagnostic consensus was based on the criteria currently in force according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and the categorization of pathologies based on the International Classification of Diseases (ICD-10). Data collection was carried out by two psychiatrists at different times, using medical records and clinical interviews.

Individuals with no history of previous and current psychotic disorder or any other psychiatric disorder, paired for age and sex, were also evaluated and selected to represent the control group (N = 924), with 489 female individuals and 440 male patients.

2.2. Study Inclusion and Exclusion Factors

The study included patients with a previous diagnosis of schizophrenia, of both genders, aged between 21 and 75 years, confirmed by clinical psychiatric evaluation and the use of assessment scales for schizophrenia (PANSS Scale, BPRS Scale, ICD-10, and DSM-5) treated at the Hospital de Clínicas Gaspar Vianna from 2005 to 2015.

Patients with bipolar depression or any other psychiatric disorder other than schizophrenia were excluded from the study, through the application of the ICD10 symptom checklist for mental disorders. All those who had acute clinical comorbidities within 3 months prior to the assessment were also excluded, who had a fever greater than or equal to 37.8 °C, and/or who had undergone immunizations in the last 4 weeks, as well as patients with chronic inflammatory diseases, such as hepatitis, cancer, diabetes, acquired immunodeficiency syndrome (AIDS), autoimmune diseases, acute infections, or in the process of pregnancy or lactation. Also, those using medication, such as corticosteroids (oral, intravenous, or topical), antioxidants, immunotherapy, anti-inflammatories and antibiotics.

2.3. Ethical Aspects

This study was carried out in accordance with Resolution 466/2012 of the National Health Council (CNS). The patients only took part in the study after being fully informed about it and signing the free and informed consent form (TCLE) and the free and informed assent term (TALE).

The research was approved by the Research Ethics Committee of Fundação Hospital Clínicas Gaspar Vianna CAAE no 41291515.0.0000.0016.

2.4. RNA Extraction and Quantification

Total RNA extraction was performed using the AllPrep Tri-Reagent kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol.

The miRNA concentrations were determined using the Qubit® 2.0 Fluorometer equipment (Invitrogen, Waltham, MA, USA), manufactured by Thermo Fisher Scientific, Waltham, MA, USA, for a final standard concentration of 5 ng/μL.

2.5. Reverse Transcriptase Reaction

Specific cDNA for the region of interest was obtained from RNA using a miRNA reverse transcriptase (RT) primer. The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) was used, according to the manufacturer's protocol. The cDNA obtained was stored at −20 °C for further analysis.

2.6. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The real-time quantitative polymerase chain reaction (qRT-PCR) was performed using the TaqMan Universal PCR Master Mix Kit (Thermo Fisher Scientific), according to the manufacturer's protocol, and the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative miRNA PCR results were normalized to U6 small nuclear RNA (snRNA; ID: 001973) as endogenous control levels. The reactions were performed in triplicate for each patient, according to the method by Gallelli et al. (2019), and incubated in 96-well optical reaction plates. The thermocycling conditions were as follows: 95 °C for 10 min and 40 cycles of 15 s at 95 °C, followed by 1 min at 60 °C. After completion of the qRT-PCR experiments, the average cycle threshold (Ct) values of triplicate reactions were determined. The comparative CT method was adopted, and as previously mentioned, U6 RNA was used as an endogenous control. The stability of the U6 endogenous control was evaluated using the computer program algorithm geNorm. The relative expression of the tested miRNAs was normalized by the mean values of CtU6 and the target miR Ct-CtU6. The difference was plotted directly as $1/\Delta ct \times 10$.

2.7. Statistical Analysis

All data were analyzed using the statistical program IBM SPSS22 (Microsoft, Redmond, WA, USA), evaluating the arithmetic characteristics, such as mean, geometric mean, standard deviation (SD), etc. Data parameters were checked for normality using the Shapiro–Wilk and Kolmogorov–Smirnov normality tests. The statistical analysis method for microRNAs was performed using Student's *t*-test with Welch correction. Graphs and tables were made using OriginPro 9.1 software. (OriginPro 9.1. Software, OriginLab Corporation, Northampton, MA, USA).

Differences were considered significant for p values < 0.05 (5%). In some analyses, because some of the values found were very small, logarithms associated with a p-value of 0.05 were used.

3. Results

The analytical model for assessing microRNAs (miRNAs) in peripheral blood, taking into account both the case and control groups, as well as the aforementioned variables, demonstrated considerable effectiveness upon the evaluation of results. Initially, a comprehensive comparison was conducted between the case group (N = 650) and the control group (N = 924), irrespective of gender and other variables. The statistical analysis revealed that all seven miRNAs (miR-34a, miR-449a, miR-564, miR-432-5p, miR-548d, miR-572, and miR-652) exhibited a p-value less than 0.05 (5%), indicating statistical significance, as illustrated in Figure 1.

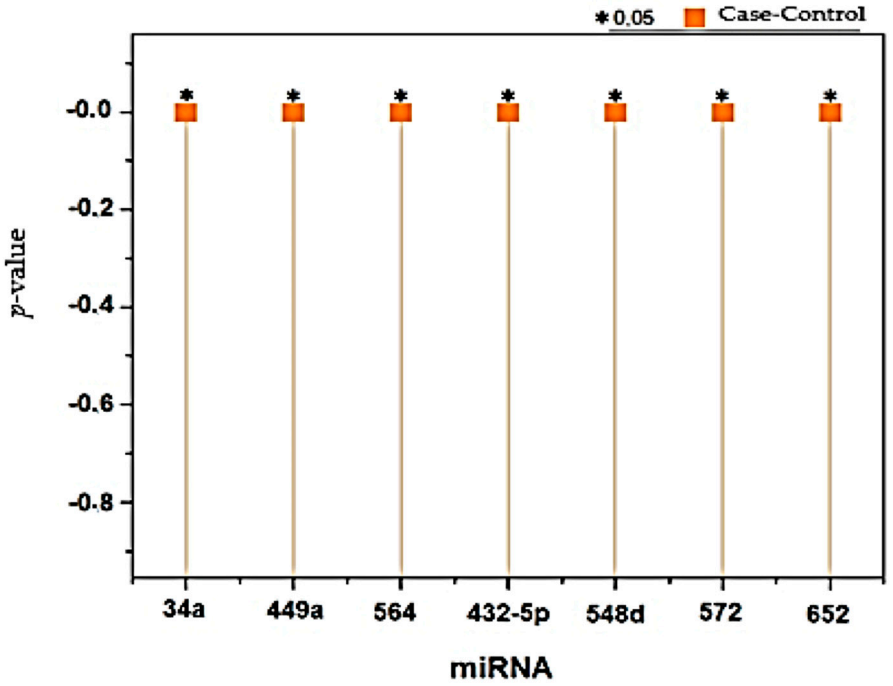

Figure 1. General graph of the case group and control group in relation to the "p-value" and miRNA. Confrontation of data from case and control groups. Statistical analysis performed by IBM SPSS22 software and graph by OriginPro 9.1 software. Data parameters were checked for normality using the Kolmogorov–Smirnov normality test and the method of statistical analysis for miRNAs was performed using Student's *t*-test with two samples at different variances, with Welch correction. Statistical differences shown are between case and control groups, with p-value < 0.05 for all miRNAs.

With respect to the variable "gender", comparisons were made separately between male patients in the case group (N = 350) and the control group (N = 435), as well as between female patients in the case group (N = 300) and the control group (N = 489). In the cohort of male patients, all seven miRNAs showed p-values below 0.05 (5%), confirming their

statistical significance. For the female cohort, utilizing the same comparative approach, it was observed that all seven miRNAs also demonstrated *p*-values less than 0.05 (5%), indicating statistical significance (Figure 2).

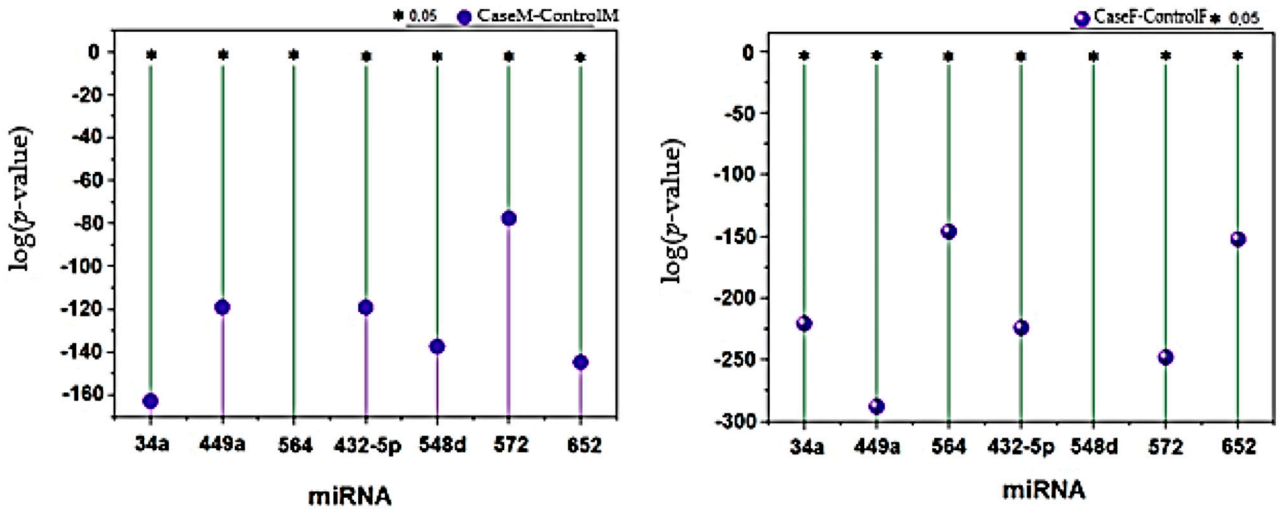

Figure 2. Graph of the case group and control group of male and female individuals, respectively, in relation to miRNA. Data parameters were checked for normality using the Kolmogorov–Smirnov normality test and the method of statistical analysis for miRNAs was performed using Student's *t*-test with two samples at different variances, with Welch correction. Statistical differences shown are between case and control groups, with *p*-value < 0.05 for all miRNAs.

When assessing smoking status within the case group (N = 650), it was found that 13.85% of individuals were smokers, while 86.15% were non-smokers. In the control group (N = 924), 21.11% of the subjects were smokers, with 78.89% identified as non-smokers. Individuals with positive smoking status were then compared with non-smokers to evaluate the impact of smoking on the expression of the seven miRNAs. The analysis confirmed that all seven miRNAs exhibited *p*-values below 0.05 (5%). Additionally, an analysis of alcohol consumption within the case group (N = 650) revealed that 34.61% of individuals regularly consumed alcohol, while 65.38% abstained. In the control group (N = 924), 16.24% reported regular alcohol consumption, in contrast to 83.76% who did not partake in alcoholic beverages. Individuals with positive alcohol status were then compared to non-drinkers to investigate the influence of alcohol on the expression of the same seven miRNAs, where all displayed statistical significance ($p < 0.05$). Regarding family history in relation to schizophrenia, the control group (N = 924) presented no individuals with a positive family history for the disorder. Consequently, the analysis focused on the case group (N = 650), wherein 20.77% had a positive family history, while 79.23% had a negative family history of schizophrenia. The analysis revealed that miRNAs 449a, 564, 432-5p, 548d, and 572 were statistically significant, as depicted in Figure 3.

Within the context of evaluating refractoriness of the disorder among individuals in the case group (N = 650), positive refractoriness was identified in 21.54% of schizophrenic patients, with 78.46% exhibiting negative refractoriness. Analogous to the variable assessing family history, miRNAs 449a, 564, 432-5p, 548d, and 572 also demonstrated statistical significance. Furthermore, analyses were conducted pertaining to the subtypes of schizophrenia, with a particular focus on paranoid schizophrenia, the most prevalent subtype, and catatonic schizophrenia, noted for its severity and unfavorable prognosis. Within the case group (N = 650), 42.31% of patients were diagnosed with the paranoid subtype, while 13.08% were classified under the catatonic subtype. In comparing the paranoid subtype to the aggregated data of the other four subtypes collectively, only miR-449a exhibited a statistically significant difference relative to all other subtypes ($p < 0.05$).

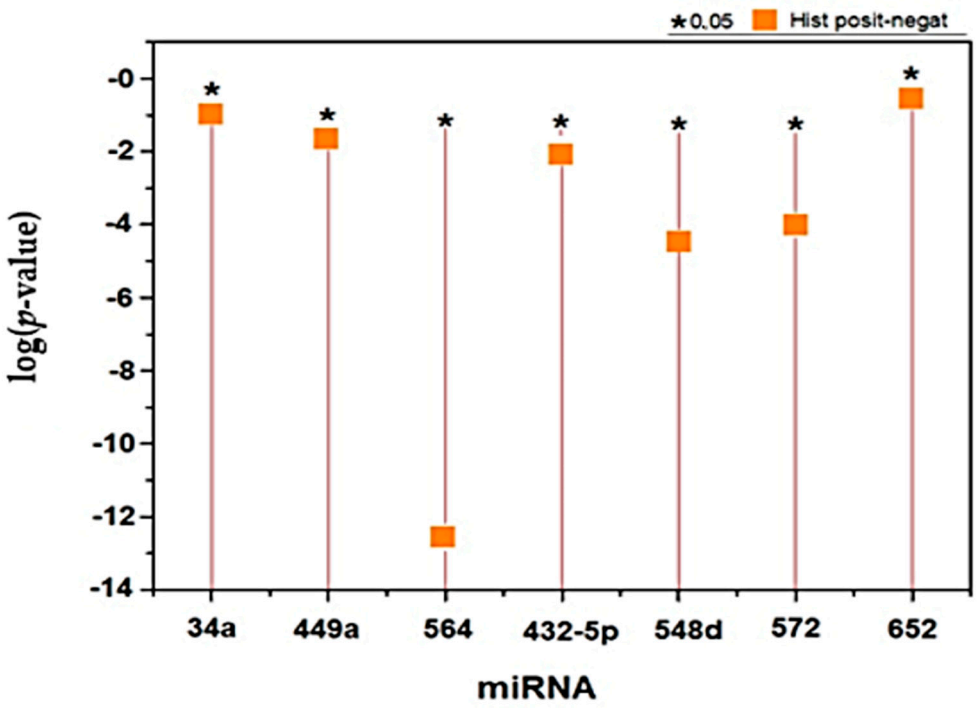

Figure 3. Graph of individuals in the case group regarding family history of schizophrenia in relation to "log(*p*-value)" and miRNA confrontation of individuals in the case group regarding family history of schizophrenia. Statistical analysis performed by IBM SPSS22 software and graph by OriginPro 9.1 software. Data parameters were checked for normality using the Kolmogorov–Smirnov normality test and the method of statistical analysis for miRNAs was performed using Student's *t*-test with two samples at different variances, with Welch correction. Statistical differences shown are between individuals in the case group, with *p*-value < 0.05 for miRNAs 449a, 564, 432-5p, 548d, and 572.

4. Discussion

Schizophrenia is a multifactorial disorder that affects millions of individuals worldwide. Studies have shown that this chronic condition presents a complex inheritance pattern involving multiple genes, biological processes, and environmental factors, with ethnicity being an important variable to consider, particularly given the significant variability in diagnosis and mortality across different continental populations [7,18]. In recent decades, epigenetics has emerged as an essential tool for understanding schizophrenia. Various non-coding RNAs have been implicated in its pathophysiology, suggesting a promising avenue for early diagnosis, treatment, and prognosis.

In this context, the present study investigated microRNAs (miRNAs) in human peripheral blood, informed by earlier studies such as those conducted by Lai et al. (2016) and He et al. (2019) [12,15]. This model is presently described in the literature as a practical, simple, and direct method for accessing individuals, facilitating the search for one or more biomarkers through a minimally invasive technique, thereby adding complementary diagnostic value to the initial hypothesis formulated by the evaluating clinician. Given the necessity for early intervention in the disease course, this model is particularly relevant for its practicality and potential for implementation at an early stage of mental disorders, as earlier therapeutic interventions following diagnosis are associated with improved prognoses.

The results indicated that the expressions of miRNAs 34a, 449a, 564, 432-5p, 548d, 572, and 652 were significantly elevated when comparing patients diagnosed with schizophrenia to healthy individuals across both genders, demonstrating statistical significance ($p < 0.05$). These findings indicate that these miRNAs may serve as potential biomarkers, either individually or in combination. In the gender-based comparison within the case group, only miR-432-5p and miR-572 did not show statistical significance ($p > 0.05$), while the remaining

five miRNAs warrant exploration within the biomarker field for schizophrenic patients. According to Sun et al. (2016) [19], miR-34a also exhibited a significant increase in patients with schizophrenia when comparing case and control groups, although no statistically significant difference was detected for miR-432 in the same cohorts. Among the analyzed miRNAs, miR-34a is arguably the most extensively documented and acknowledged by the scientific community for its role in neurogenesis [20] and processes related to neural differentiation [21]. Its expression has also been found to be altered in various psychiatric disorders, including schizophrenia [15], bipolar disorder, and major depression [22].

Concerning the interplay between schizophrenia and lifestyle habits, it is recognized that individuals who chronically consume alcohol and have previously exhibited psychotic symptoms, particularly hallucinations, face a 5–30% increased likelihood of developing schizophrenia [23]. Regarding smoking, a complex relationship exists between nicotine and schizophrenia, with some evidence suggesting a slight improvement in alertness levels amongst smokers with schizophrenia; however, this benefit is minimal when contrasted with the adverse health consequences of smoking [24].

As for correlating miRNA expression in schizophrenic patients with these harmful habits, the current literature is lacking studies that connect the expression of the seven miRNAs analyzed in this research with substance use in these patients. However, the present study found a statistically significant difference ($p < 0.05$) between the case and control groups regarding the variable "smoking", indicating an elevation in the expression of all seven miRNAs. Furthermore, when comparing the two populations within the case group (smokers and non-smokers), the same results were obtained, as all seven miRNAs were more highly expressed among smokers, achieving statistical significance ($p < 0.05$). Similarly, the variable "alcohol" demonstrated an increase in the expression of the seven analyzed miRNAs between the case and control groups. In comparing alcohol users and non-users within the case group, only miR-449a exhibited a statistically significant alteration in expression ($p < 0.05$).

It is well established that a family history of schizophrenia poses a significant risk factor, particularly when first-degree relatives are affected by the disorder. Numerous studies have demonstrated that genetic risk variants inherited by a patient with schizophrenia directly correlate with certain miRNAs. For instance, the study by Bigdeli et al. (2020) [25], which examined 33,422 individuals divided into schizophrenic and healthy groups, analyzed the polymorphisms present in their genotypes. However, no corresponding studies were found in the literature linking the seven miRNAs evaluated in this study with expressions correlated to an individual's family history.

Nonetheless, the present study aimed to explore the potential relationship between the seven miRNAs and family history within the case group. When comparing individuals with schizophrenia who had positive and negative family histories of the disorder, miRNAs 449a, 564, 432-5p, 548d, and 572 exhibited statistical significance ($p < 0.05$) between the groups.

To assess refractoriness to antipsychotic treatment (defined as inadequate response to the first antipsychotic), an important consideration that affects approximately 30% of patients with schizophrenia [26], the miRNAs were also correlated with this variable within the case group. Among the 650 subjects in the case group, 140 were identified as refractory to treatment. The same five miRNAs (449a, 564, 432-5p, 548d, and 572) that demonstrated differences in relation to family history were also significantly different between the refractory and non-refractory groups ($p < 0.05$). The study by You et al. (2020) [27] similarly evaluated miRNAs in refractory schizophrenia patients through peripheral blood analysis. Among the 34 miRNAs analyzed in their case and control groups, only miRNA 432-5p was common to both studies, where You et al. found an increased expression (up-regulation) of miR-432-5p in the refractory patient group.

The assessment, grounded in the current International Classification of Diseases (ICD-10), also enabled an expansion of the variables considered within the case group, thus allowing for the study of statistical differences among the five most prevalent subtypes of schizophrenia: paranoid, disorganized (also known as hebephrenic), catatonic, residual,

and undifferentiated. Most studies exploring epigenetic correlations with schizophrenia centralize their case evaluation on the paranoid subtype, given its predominance and higher incidence. Ghazaryan et al. (2019) [28] examined various miRNAs in patients with paranoid schizophrenia, including miR-31, 146a, 181c, and 155, reporting increased expression among paranoid patients compared to their healthy counterparts. However, no studies were identified that addressed the remaining schizophrenia subtypes in relation to the miRNAs analyzed in this research.

This study, conversely, examined the relationships among the subtypes based on the case group. When comparing the paranoid subtype to the aggregate of the other four subtypes, only miR-449a exhibited a statistically significant difference across all ($p < 0.05$). The disorganized subtype demonstrated significant relevance for miR-432-5p and miR-548d relative to other subtypes; the catatonic subtype revealed significance for miR-449a, miR-548d, and miR-652; the undifferentiated subtype showed significance for miR-34a and miR-548d; finally, the residual subtype did not indicate any statistically significant miRNA in relation to the others, only isolated significances with specific subtypes.

5. Conclusions

The findings of this study indicate that the miRNAs explored (miR-34a, 449a, 564, 432-5p, 548d, 572, and 652) may serve as potential biomarkers for schizophrenia, as they demonstrated significant differences among the studied groups under various clinical circumstances. Upon developing diagnostic hypotheses and considering schizophrenia as one of them, investigators are positioned to determine which miRNAs to investigate, based not only on diagnostic criteria but also on pertinent variables such as gender, tobacco and alcohol consumption, family history of schizophrenia, treatment refractoriness, and supplementary laboratory tests aimed at clarifying the specific subtype of the disorder. Therefore, we advocate for the continuation of studies to validate the results obtained in this investigation and further elucidate the biological roles of the miRNAs assessed in the context of schizophrenia diagnosis.

Author Contributions: Conceptualization, A.L.d.S.R. and R.M.R.B.; methodology, C.d.C.S., D.D.F.Á.A., and A.C.-P.; validation, D.D.F.Á.A. and A.C.-P.; formal analysis, C.d.C.S.; investigation, A.L.d.S.R. and R.M.R.B.; resources, A.L.d.S.R. and R.M.R.B.; writing—original draft preparation, A.L.d.S.R.; writing—review and editing, D.D.F.Á.A. and A.C.-P.; visualization, A.C.-P. and M.M.B.G.I.; supervision, R.M.R.B. and M.M.B.G.I.; project administration, R.M.R.B.; funding acquisition, A.L.d.S.R. and R.M.R.B. All authors have read and agreed to the published version of the manuscript.

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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 Fundação Hospital Clínicas Gaspar Vianna (protocol code 41291515.0.0000.0016 approval Date 20 January 2015).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to containing information that could compromise the privacy of research participants.

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Conflicts of Interest: The authors declare no conflicts of interest.

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