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Title

Towards a MicroRNAs-Based Biomarker Panel for AIS: A Meta-Analysis

Running title

Towards a microRNAs-based biomarker panel for AIS

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Conflict of Interest Statement

The authors declare NO potential conflict of interest.

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Abstract

Background: Acute ischemic stroke is among the main causes of mortality worldwide; a rapid and opportune diagnosis is crucial to improve a patient's outcome. MicroRNAs are quite useful for a rapid and accurate diagnosis.
Methods: We perform both structural networks approach and a meta-analysis (using a random-effect model to evaluate the heterogeneity and risk bias, according to the PRISMA statement) to analyze the feasibility to develop a microRNA-based biomarker panel for an opportune AIS diagnosis.
Results: Structural networks identify a set of eight miRNAs (miR-16, miR-124-3p, miR-484, miR-15a, miR-4454, miR-107, miR-125b-5p and miR-320b) as preliminary microRNA-based biomarker panel, from these only three microRNAs are significantly associated with the main risk factors of AIS, (miR-107: hypertension, 95% CI 9.74-53.24 $p<0.0001$, type 2 Diabetes mellitus, 95% CI 2.18-19.26); $p=0.0008$; miR-16 hypertension, 95% CI 1.26-3.56 $p=0.0046$, smoking, 95% CI 1.07-3.54 $p=0.0277$; and miR-15a hypertension, 95% CI 1.26-3.56 $p=0.0046$; smoking, 95% CI 1.07-3.54 $p=0.0277$). However, the meta-analysis reveals that data is highly heterogeneous and biased; and only microRNAs isolated from plasma samples and further processed in microarrays are the most reliable to distinguish AIS patients.
Conclusions: Together our results show that although there are some miRNAs that seem to be associated with AIS, we are still far to develop a miRNA-based biomarker for AIS diagnosis and it is necessary to harmonize the protocols, results and include more populations for further studies otherwise we will remain throwing punches in the dark.

Keywords: miRNAs, stroke, acute ischemic stroke, biomarkers, meta-analysis.

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1. INTRODUCTION

Brain stroke is a major public health problem worldwide that could be divided into hemorrhagic and ischemic. The latter one in turn could be subdivided into transient stroke and acute ischemic stroke (AIS); which represents the 80-90% of cases all-over the world[1]. Additionally, data from the World Health Organization and from the Institute of Health Metrics and Evaluation, mentioned that AIS is the second cause of morbidity, incapacity, and mortality in individuals over 60 years old[2].

The AIS results from the permanent local blockage in the arteries that supplies glucose and oxygen into the brain, that required of a rapid evaluation and treatment to achieve best outcomes[3,4]. Both endovascular and thrombolytic (recombinant tissue plasminogen activator, rtPA) therapies helps to the restoration of cerebral blood flow, however, rtPA administration induces symptomatic intracerebral hemorrhage in 3% of AIS-patients[5], thus, clinicians are very cautious with its administration to avoid health complications.

Additionally, AIS diagnosis is quite challenging since it could be confused with other types of stroke that need other type of treatments, this added to other factors such as deficits on the efficient triage of the emergency rooms, expenses and availability of experts, neuroimaging equipment and a scarce education among the general population to identify opportunely stroke, narrow the window of time to manage adequately the AIS[6,7]. Hence AIS diagnosis should be more efficient through the development of non-invasive, cheap, and highly sensitive strategies.

Recently, several studies have been focused on characterizing biomarkers[8] to differentiate among the most common stroke subtypes accurately. The non-coding RNAs such as microRNAs (miRNA´s, small single-stranded non-coding RNA molecules from

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~22 nucleotides which are endogenously expressed and regulate gene expression though different epigenetic mechanisms) have been proposed as novel biomarkers since they are highly stable and differentially expressed for specific conditions such as cancer, arthritis, osteoporosis, infectious diseases, cardiovascular diseases, neurodegenerative diseases, and AIS[9–11]; miRNA´s are easily isolated from different liquid biopsies such as whole blood, plasma, serum, blood circulating exosomes, peripheral blood cells, or cerebrospinal fluid[12] with low invasiveness for the patients, additionally, it they could be easily measured with conventional labs with the minimum requirements on molecular biology[13-17].

Particularly, the research in AIS offers several studies characterizing miRNA´s profiles, which differentiate accurately from other stroke types[18-22]. However, there still a lack of consensus that may constitute a miRNA-based biomarker panel for AIS diagnosis. Therefore, in the present study we performed a structural network analysis followed by a traditional meta-analysis to identify whether among the studies published from 2015 to 2020 is possible to suggest a feasible miRNA-based biomarker panel for AIS diagnosis.

2. Methods

2.1 Study strategy and Selection

We follow the PRISMA statement[23] to perform this study, methods are submitted to the PROSPERO database with registration number **CRD42020206145**. The studies were explored on the SCOPUS database using MESH terms *microRNAs* AND *mirna* AND

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acute ischemic stroke OR *brain stroke*. All relevant studies from 2015 to 2020 were searched in the Scopus database in July 2020 by two independent reviewers.

Selected studies fulfil the following eligibility criteria:

2.1.1 *Inclusion criteria:*

1. Studies reported or published between 2015 and 2020.
2. Studies that discuss miRNAs differentially expressed in AIS.
3. Studies were presenting both cases and controls groups.
4. Studies performed in human samples such as whole blood, serum, plasma, exosomes, or blood cells; and did not exclude studies based on the ethnicity of study participants.
5. Only studies that validate AIS diagnosis by neuroimaging, such as computed tomography (CT) or magnetic resonance imaging (MRI), were included.
6. Studies conducted within 24 h of AIS symptoms.

2.1.2 *Exclusion criteria:*

1. Studies published in other languages different from English.
2. Narrative reviews, intervention studies, letter to editors, and non-original articles.
3. Unpublished data, incomplete datasets, or preprints.
4. Studies without available data.
5. Studies without controls.
6. Studies using duplicated data.
7. Studies performed in vivo.

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8. Studies performed in vitro, even when these were human-derived.
9. Studies performed with already published databases.

2.2 Data extraction

We retrieved relevant information from each selected study as depicted in Table 1S from the supplementary material.

2.3 Network analysis

Structural networks were built using Cytoscape software v 3.8[24]. The most connected genes among the network were identify using the Cytohubba plugin[25]. Additionally, we use BinGO plugin of Cytoscape[26] to identify the principal signaling pathways altered by the miRNAs. The most significantly enriched pathways ($p < 0.05$, p-values) were corrected using the Benjamin-Hochberg procedure, according to previous reports[27].

2.4 Associations with clinical variables

The association between miRNA expression and the most common risk factors associated with AIS development (HTN, T2DM or smoking) were calculated by the odds ratio (OR) for the differentially expressed (DE) miRNAs that appear shared among tissues or geographical regions. The cases were considered individuals having AIS and one risk factor simultaneously; and the controls were the remaining individuals without AIS. Thus, we considered three types of cases, and consequently, three types of OR are 1) AIS in patients with HTN + DE-miRNA, 2) AIS in patients with T2DM + DE-miRNA, or 3) AIS in patients being active smokers + DE-miRNA.

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2.5 Meta Analysis

We calculated the heterogeneity using χ^2 -tests based on the Q-test and the I-squared (I^2) statistical tests. The pooled effect size (OR) was assessed based in the random-effect model, if heterogeneity was considered statistically significant (I^2 -value more than 50% and $P < 0.05$). To evaluate the specific effects, we also performed subgroup analyses of the data arranged by tissues, geographical origin, and technological platform. The complete meta-analyses of the retrieved data were performed using the *Metafor* R package in R-studio (Version 3.4), according to the methodology previously reported by [28]. The complete description of the algorithm is in http://www.metafor-project.org/doku.php/metafor.

3. Results

We identify 678 different studies by applying the filters mentioned above. From these, we use 25 studies to construct the networks. According to the PRISMA statement, Figure 1 depicts in detail the article selection for the subsequent analyses.

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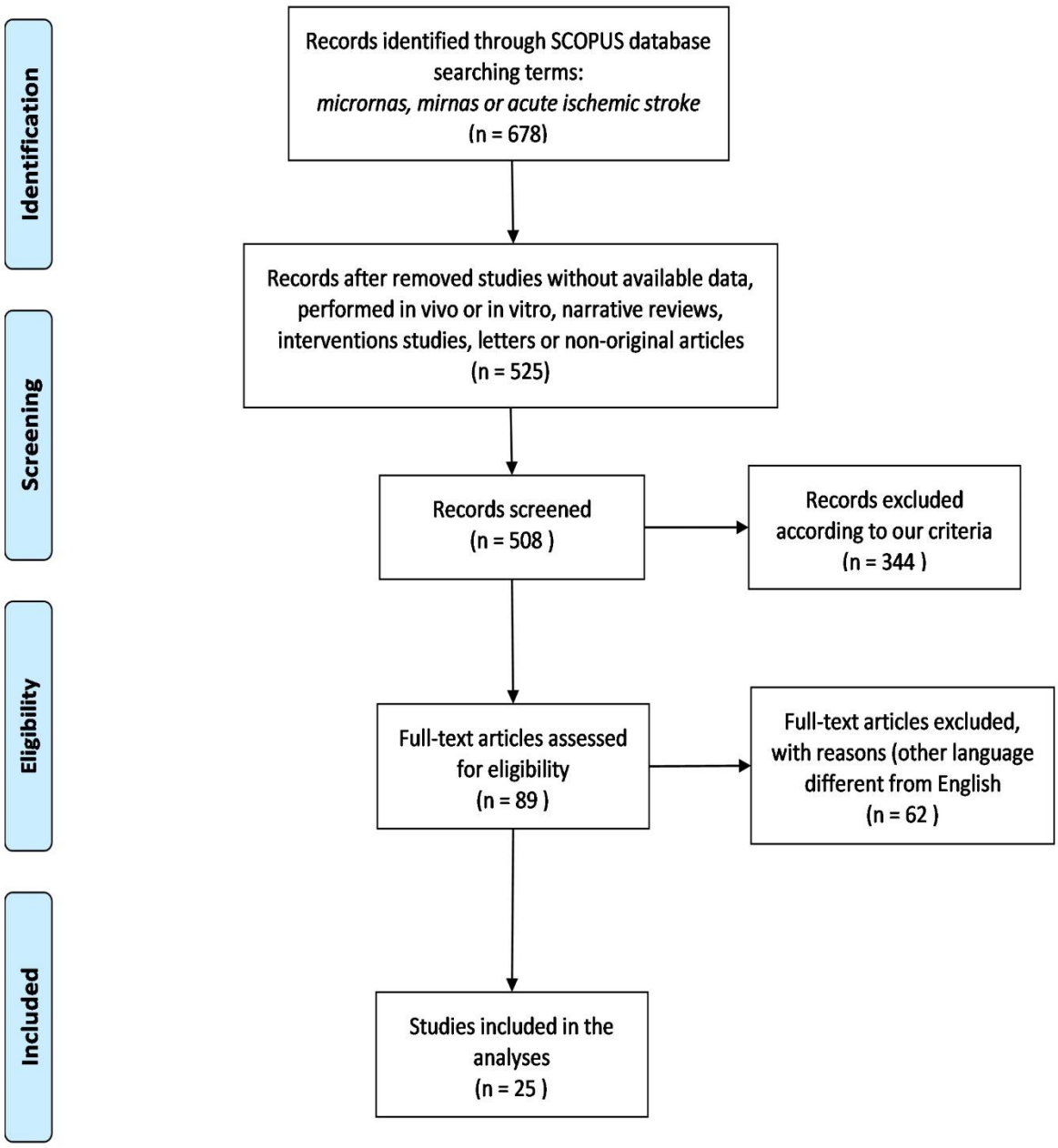

Figure 1. Flow diagram of article selection for the meta-analysis. Modified from [23]

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Then we build a network (Figure 2) which demonstrates that despite a significant amount of the miRNAs identified on blood, plasma, CSF, serum, exosomes, and immune cells, only six miRNAs are shared among at least two of them (miR-16, miR-124-3p, miR-484, miR-15a, miR-4454, and miR-107). Interestingly, most miRNAs are upregulated, and plasma is the tissue that shares most of the targets; thus, it could be considered a hub in the network and suggest that plasma may be enriched in miRNAs compared to the other tissues .

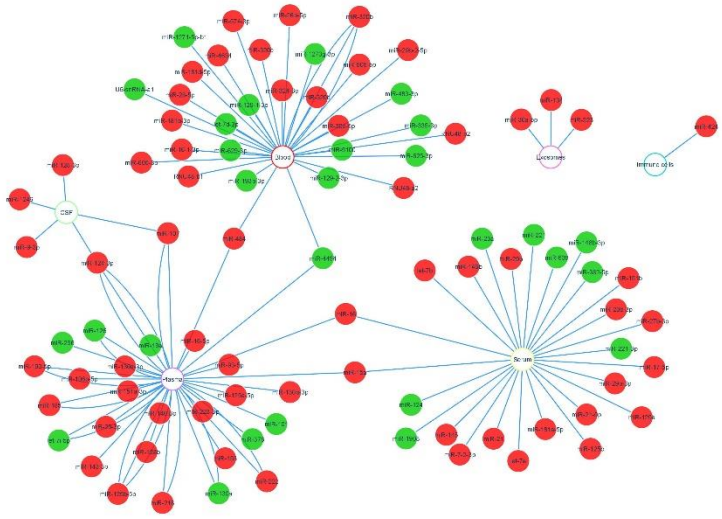

Figure 2. Structural network of the DE miRNAs derived from AIS patients according to sample origin. This network organizes miRNAs (targets) according to the tissue (source) from which the samples derived (complete data are in Table 1S). DE miRNAs appear in red (upregulated) or green (downregulated) according to their expression; multiple edges indicate the number of independent studies that also report this target. Six miRNAs appear at the network center, representing that these are common in at least two different tissues.

Additionally, we try to answer which miRNAs are shared among the countries where the studies belong, so we built another network that depict such issue. The network from Figure 3 identifies that five miRNAs (miR-125b-5p, miR-320b, miR-124-3p, miR-484, and miR-107) shared among China, Denmark, Germany, and the USA. However, Spain stands apart from the network with the miR-638.

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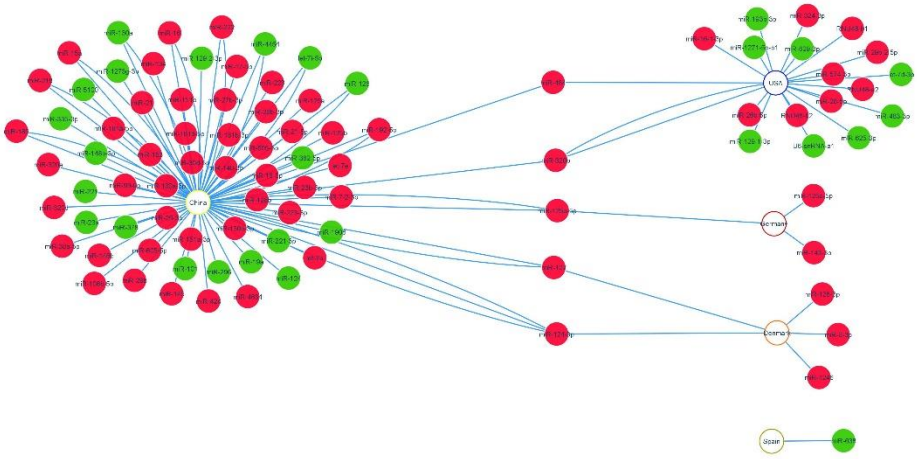

Figure 3. Structural network analysis of miRNAs differentially expressed in AIS from human samples according to the geographical distribution. Data were extracted from 25 selected articles in agreement with our inclusion criteria (Supplementary Information Table 1S). Network was built by using Cytoscape software (v.3.8.0). Nodes correspond to miRNAs (targets-according to its expression those appear in red (upregulated) or green (downregulated)) and countries (sources). Multiple edges indicate different studies joining the same miRNA with the same country.

miRNAs resulting from Figures 2 and 3 were submitted miRNet database (https://www.mirnet.ca/ accessed March 2021) to identify the genes that such miRNAs alter. Then, with the Cytohubba plug-in we identify the most connected nodes (genes) (CREBF, TUBB, CDK6, ABL2, ELK4, GANAB, FBXL18, CALU, CCNE, TNRC6B, and HNRNPA2B1) in the network (Supplementary material Figure 1S). Besides, the gene enrichment using the KEGG, GO:BP, Reactome databases and the BinGO plugin showed that miRNAs in AIS altered genes that are significantly associated to neurotrophin signaling pathway, cell cycle, cell adhesion, protein folding, apoptosis, angiogenesis, aging, oxidative stress, and mitochondrial membrane organization signaling, among others (Supplementary material Figure 2S).

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Since HTN, T2DM, dyslipidemias, or being an active smoker are often associated to AIS occurrence, we estimated the association between the selected DE miRNAs identified by the networks in AIS and individuals with has HTN, T2DM, or being an active smoker. The results demonstrate that miR-107, miR-16 and miR-15a overexpression is significantly associated with individuals with both AIS and HTN (OR= 22.77 95% CI 9.74-53.24 p<0.0001, OR=2.12 95% CI 1.26-3.56 p=0.0046, respectively, Figure 4 A-C). Likewise, miR-107 is significantly associated with AIS in T2DN (OR= 6.48 95% CI 2.18-19.26; p=0.00277), and both miR-16 and miR-15a are significantly associated with AIS in active smokers (OR= 1.95 95% CI 1.07 -3.54; p=0.00277), suggesting such miRNAs as potential biomarkers of AIS. Complete data from the association analysis appear in Table-2S.

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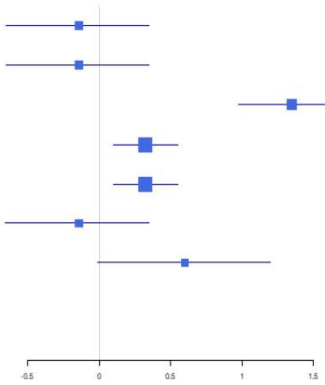
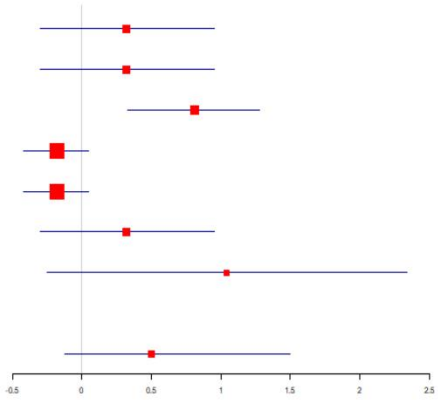
A

miRNA	AIS+miRNA Hypertension	Controls Hypertension	OR
miR-484	22	17	-0.14
miR-107	22	17	-0.14
miR-107_2	92	9	1.35
miR-16	64	43	0.32
miR15a	64	43	0.32
miR-4454	22	7	-0.14
miR-125b-5p	16	10	0.6
Summary			0.61



B

miRNA	AIS+miRNA T2DM	Controls T2DM	OR
miR-484	8	3	0.32
miR-107	8	3	0.32
miR-107_2	37	4	0.81
miR-16	38	55	-0.18
miR15a	38	55	-0.18
miR-4454	8	3	0.32
miR-125b-5p	4	0	1.04
Summary			0.53



C

miRNA	AIS+miRNA Smoking	Controls Smoking	OR
miR-484	9	9	-0.5
miR-107	9	9	-0.5
miR-107_2	49	17	0.25
miR-16	36	25	0.29
miR15a	36	25	0.29
miR-4454	9	9	-0.5
miR-125b-5p	8	9	-0.09
Summary			-0.01

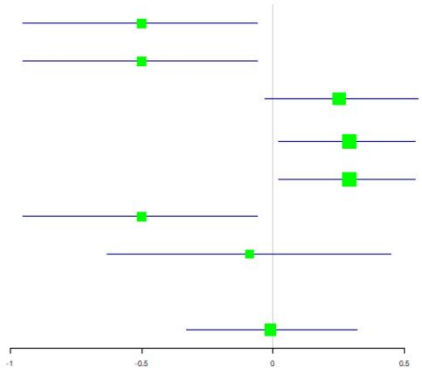


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Figure 4. Forest plot for miRNA associations with risk factors. Representative forest plot of the associations (OR) between the eight miRNAs shared among different tissues and geographical regions in individuals experiencing AIS simultaneously and (A)HTN (blue), (B)T2DM (red), or (C) being active smokers (green).

Concomitantly, we aimed to test the potential heterogeneity and the publication bias of the studies, to confirm our results from the network analysis and to suggest a reliable biomarker panel for AIS diagnosis. To achieve such a goal, we performed a meta-analysis with the data retrieved from 25 selected studies. Surprisingly, the results (Figure 5A) shows that there is no statistically significant difference between the DE miRNAS in AIS patients and controls (p-value = 0.0943), suggesting that the overall analysis of the raw data is not useful enough to differentiate between patients with AIS or controls. In addition, these results also show significant heterogeneity (Q= 10588.83, p-value <0.0001), high risk of publishing bias (Figure 5B), and high sampling variance (Figure 5C); probably due to the lack of consensus in protocols focused on characterized miRNAs expression in AIS patients.

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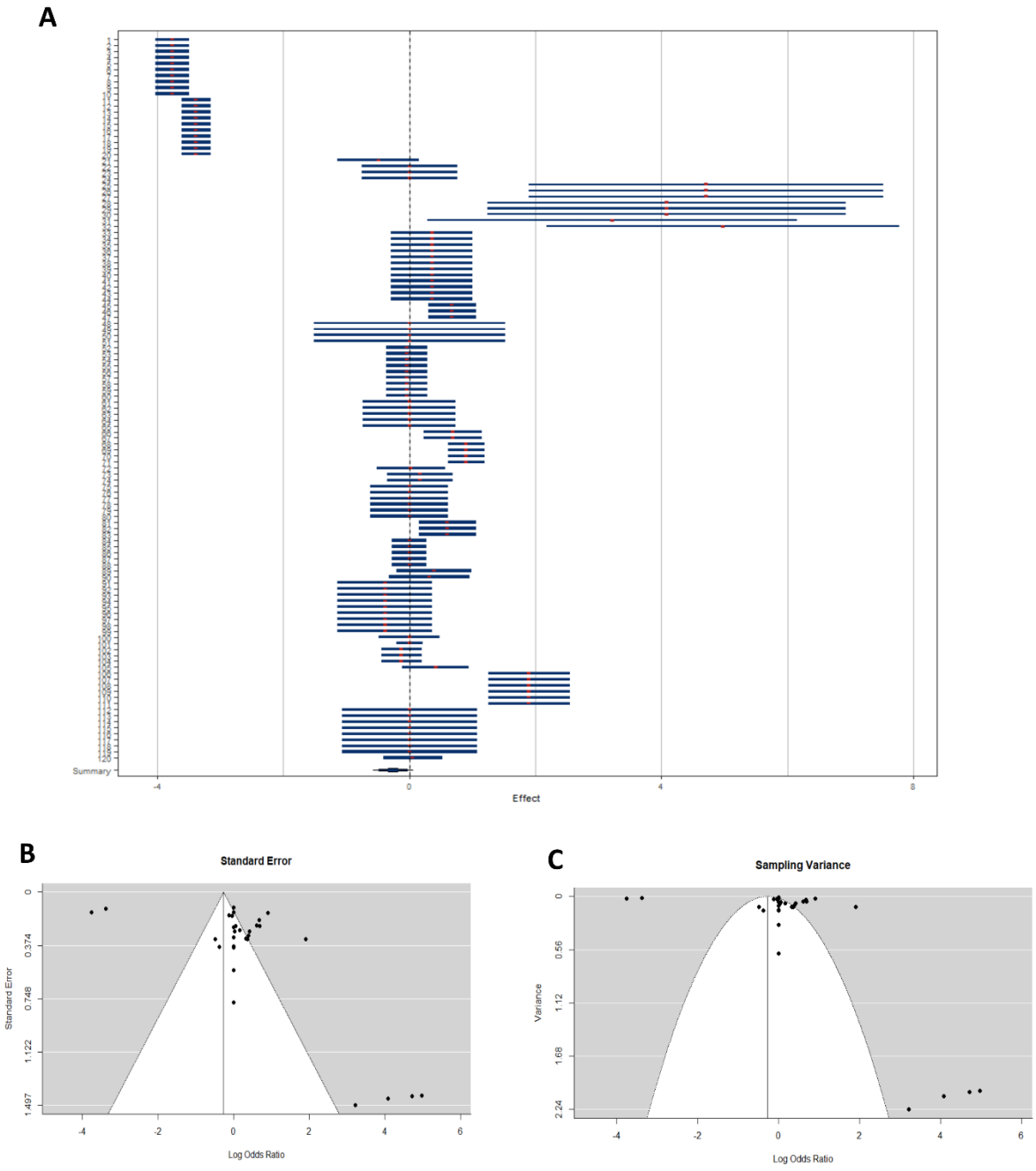

Figure 5. Meta-analysis of all the miRNAs retrieved from the 25 selected studies. (A) Forest plot for the differentially expressed (DE) miRNAs retrieved from the studies summarized in Tables 1S. The scattering shows two groups of miRNAs, ones that are positively associated with AIS and others that are not (associated with the controls, No-AIS). However, globally the results are not statistically significant (p-value 0.0943). (B) Funnel plot for all the DE miRNAs. Each dot represents a miRNA retrieved from the database from Table 1S. The outliers in the grey region represent the miRNAs that have publication bias, since they are out of the significance region (95% CI). (C) Sampling variance plot demonstrates

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that the analyzed miRNAs have a significant variability suggesting that there is heterogeneity in the sampling.

Due to the results above, we tested whether meta-analysis among subgroups could be useful for AIS diagnosis. The results show that blood (Q= 1823.75 p-value <0.0001), plasma (Q= 111.92 p-value <0.0001), and serum (Q= 207.91 p-value <0.0001) are a reliable source of miRNAs for AIS in comparison with the data from CSF (p-value=1) and other (immune cells and exosomes, p-value= 0.07). Nevertheless, the results from the funnel plots reveal that except for the plasma-derived samples, the rest of the tissues have bias, probably due to the sampling variance (Figures 3S-5S).

Likewise, when data is analyzed by geographical regions the results demonstrate that only the studies performed in China (p-value <0.0001) and USA (p-value <0.0001) can identify AIS patients from the controls. However, the studies performed in both Europe and USA are significantly biased; in contrast to the data retrieved from the studies performed in China probably due to the small sampling variance (Figures 6S-8S)

Finally, our results also suggest that among the available platforms to analyze miRNAs expression, microarrays are significantly useful to identification DE miRNAs in AIS patients (p-value=0.007, Figures 9S-11S).

4. Discussion and Conclusions

Despite the great technological advances in neuroimaging, both CT or MRI have limitations such as sensitivity in early stages of AIS, technical issues to view some brain regions, expensive costs, low equipment availability and expertise needed to distinguish among stroke subtypes; that unfortunately are often common in the public health centers,

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and that delay both the opportune AIS diagnosis and treatment. Hence, developing novel strategies as molecular biomarkers may allow the efficient and accurate diagnosis of AIS. The research on biomarkers for AIS diagnosis or prognosis is an active and dynamic field that have been nourished from several studies worldwide, particularly those based on miRNAs profiles that seems to be a useful tool for the development of a reliable biomarker panel to distinguish among stroke subtypes [29–31]. Also, since the research on this field increases daily a meta-analysis is the best way to move forward and offer a potential biomarker-panel for AIS diagnosis.

At first sight our results show a potential set of miRNAs that may be used for AIS characterization, such miRNAs are involved in biological processes (excitotoxicity, neuronal death, inflammation, neurogenesis, and angiogenesis) often common during AIS [32]. However, when we performed the metanalysis, we found that such panel are poorly feasible since data have high risk of publication and sampling bias. Despite such observation when data were analyze per subgroup we found that that further studies seeking to characterize miRNA-based biomarkers for AIS diagnosis may be conducted in plasma, since this shows the lowest risk of publishing bias of all the analyzed tissues, as previously reported [33,34].

Similarly, when data is analyzed by the geographical, the results show that despite the heterogeny of the data only China exhibits twenty-one miRNAs with both desirable features, they are statistically significant and with the lowest risk of publishing bias, from these miRNAs only five (miR-124-3p, miRNA-125b-5p, miR-107, miR-221-3p, and miR-

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16) can distinguish AIS patients from controls. Nevertheless, since the largest number of studies derive from China this could represent a drawback for our analysis, so further analyses are required to validate these miRNAs in other populations.

Since the results from the last meta-analysis demonstrate that microarrays are significantly suitable to discriminate samples derived from AIS patients or from controls and have low risk of publishing bias; this technology may be considered as one of the best options to perform further studies that seek to characterize miRNAs profiles for AIS. These results are probably because most companies use the same databases to build microarrays, and in consequence the variance among the results decreases significantly. Despite these promising results, this technology does not solve the issue that represents the narrow window of time for AIS diagnosis; so, we should delve into other technologies that combine specificity, sensitivity, and efficiency for AIS diagnosis.

Notwithstanding, our study have limitations such as the number of studies included such limitation correspond to the heterogeneity and lack of relevant clinical data, including a complete survey about individual´s lifestyles, the heterogeneity in protocols, sample collection, miRNA isolation methodologies, and the platforms used for miRNAs analysis. In addition, the lack of representation of Latin-American population, also contributes somehow to biasing the results. Hence, we suggest that further validation of the proposed miRNAs set is urgently required in other cohorts of AIS patients; likewise, it is necessary to work in harmonized protocols that help to decrease the heterogeneity. Despite such limitations, our study offers a robust perspective about the molecular analysis performed

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at date on miRNAs and biomarkers for AIS diagnosis, this study also offers a complete insight about the crucial targets in AIS, providing a new direction for further therapeutic interventions to decrease the consequences of such conditions.

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6. Supplementary material

Table.1S. Complete data retrieved from the 25 selected studies.

Tables.2S. Complete data from the association analyses.

Fig 1S and 2S. Gene enrichment analysis and Pathway enrichment analysis.

Fig.3S-11S. Complete data from the metanalysis

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