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Diagnostic accuracy of cerebrospinal fluid and serum-isolated extracellular vesicles for glioblastoma: a systematic review and meta-analysis

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Abstract

Background: Glioblastoma (GBM) is the most malignant brain cancer because there are no available biopsy-free methods for the diagnosis or the preoperative early detection of GBM. In this regard, the development of a non- or minimally invasive method for early detection could increase the survival of

GBM patients. **Methods:** The present study aimed to assess the diagnostic accuracy of extracellular vesicles (EVs) derived RNAs, isolated from patients' CSF or serum for GBM diagnosis. For this purpose, we searched all literature databases and performed a backward and forward reference checking procedure to retrieve appropriate studies. We conducted a meta-analysis on EVs derived biomarkers as well as sensitivity analysis and meta-regression. **Results:** We identified EVs-derived 24 RNAs, which can diagnose GBM. The analyzed pooled data showed 76% sensitivity, 80% specificity, and 0.85 AUC, for 16 biomarkers. Besides, the pooled PLR, NLR, and DOR were 3.7, 0.30, and 12, respectively. Subgroup analysis did not show a significant difference between serum and CSF. **Conclusions:** According to the pooled sensitivity, specificity, and AUC for EVs derived biomarkers, and we suggest that EVs-derived biomarkers might serve as a high potential and non-invasive diagnostic tool for GBM detection using serum and CSF samples.

Keywords: Biomarkers, Diagnosis, Exosomes, Extracellular vesicles, Glioblastoma, Meta-analysis, Systematic review

Article highlights:

1. Extracellular vesicles derived RNA biomarkers could diagnose glioblastoma with 76% sensitivity and 80% specificity.

2. The overall AUC for extracellular vesicle-derived biomarkers for the diagnosis of glioblastoma is 0.85.

3. There are no significant differences in diagnostic accuracy between serum- and CSF derived EVs for glioblastoma.

1. Introduction

Glioblastoma, also known as glioblastoma multiforme (GBM), is a grade-IV brain cancer and the most malignant type of glioma [1,2]. The traditional treatment of GBM is the tumor resection followed by radiotherapy and chemotherapy. This type of treatment has limited effectiveness due to high rates of relapse, overall resistance to therapy, and serious neurotoxicity and neurological side effects [3,4]. Also, the prognosis of GBM is often very poor due to the lack of early detection and personalized treatments [5]. Since diagnosis with a tissue biopsy, especially in brain cancers, is a highly invasive, expensive, and time-consuming procedure, it is essential to develop a non-invasive, affordable, and efficient method for the detection and grade prediction of cancers. In this regard, identification of biomarkers with high diagnostic accuracy in the patients' different body fluids, especially CSF and serum, can enhance the early diagnosis of GBM and other neurological cancers and diseases [5].

In recent decades, many studies have aimed to introduce novel, accurate, and specific biomarkers for different types of cancers [6-8]. One new platform for the diagnostic biomarker discovery is extracellular vesicles (EVs), such as exosomes and microvesicles. EVs are heterogeneous populations of vesicles classified into three groups: exosomes, microvesicles, and apoptotic bodies. Exosomes are the seemingly homogeneous fractions of EVs with 30-150 nm in size [9]. Regarding the origin, exosomes are distinct from other types of EVs, including microvesicles (with heterogeneous size from 50 to 1000 nm in diameter) and apoptotic bodies [10,11]. Used as biomarker sources, microvesicles, and exosomes play important roles in cellular communication [12]. In the literature, there are some problems in the terminology of EVs, exosomes, and microvesicles. According to ISEV2018 [13], different fractions of EVs have different characteristics in terms of their size and surface markers [14]. Obtaining a pure population of

exosomes and microvesicles is a very important step in diagnostic studies and requires some criteria and characterization protocols. Unfortunately, some studies do not meet these criteria. Therefore, in this study, we will use the general term (EVs) for both exosomes and microvesicles to avoid further mistakes by the readers.

EVs carry various extracellular biomolecules, some of which are indistinguishable in the corresponding biological fluid as cell-free or naked molecules [15]. This feature can provide various sensitive and specific diagnostic biomarkers for different pathophysiological conditions [11]. In the beginning, Valadi et al. stated that small-sized EVs (exosomes), in addition to proteins, possess different types of RNAs [16]. Today, it is well known that EVs can transport diverse molecular constituents, such as lipids, proteins, and nucleic acids [10]. Important findings on the biological nature of EVs and their contents implied that EVs contents could be used as a tool for discriminating cancerous cells from healthy cells [17]. Further studies demonstrated that the amount of EVs, particularly exosomes in various cancer patients, is higher than in healthy persons [18]. According to the recent studies, biomarkers derived from EVs show similar or higher specificity and sensitivity compared to other circulating biomarkers. As stated earlier, technical improvements for EVs isolation could bring EVs derived biomarkers as a new platform for the diagnosis of cancers and other diseases [17,19].

To the best of our knowledge, there has not been any systematic review articles on the assessment of the EVs derived RNA biomarkers for GBM diagnosis. Thus, the current study aimed to review and analyze the primary studies on the diagnostic accuracy of EVs for GBM and provide a pooled diagnostic data of these studies to clear the situation for future studies and possible clinical application.

2. Methods

We designated a protocol following PRISMA guidelines to report a systematic review and protocol for Reviews of Diagnostic Test Accuracy [20]. Before the publication of the study, we submitted this systematic review on PROSPERO on 26 Jun 2019 (registered on 17/09/2019 with CRD42019132438 ID).

2.1. Search strategy designing

To retrieve all possible studies in the area of our study purpose, we aimed to develop a full search strategy. We tried to implement a comprehensive systematic search that combined (using the Boolean Operator) text-words and subject headings (MeSH or equivalent) of the following electronic databases: PubMed (including Medline), Embase, Web of Science, Scopus, and ProQuest (as databases for grey literature) through December 2019. The search was performed according to the search strategies stated in the protocol Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy [21]. For the search in electronic databases, we used all possible keywords related to the "Extracellular vesicles" and "Glioblastoma" , which were extracted from the MeSH database and Emtree. This strategy of search allowed us to conduct a comprehensive search by a recently published protocol [22]. The databases were searched without any restrictions.

The literature search was performed independently by two investigators (AT and DJ). Any possible discordance was compared to that of an additional investigator (YM). A reference checking was conducted for the published reviews adapted to our title in order to retrieve any missed related articles through database searching. Similar to the screening of online database results, the references of all these articles were screened manually. This step ensured that all

studies relevant to the diagnostic accuracy of EVs-RNAs and detection of GBM are included in our final library. The full search strategy was shown in Table S1.

2.2. Data extraction and management

2.2.1. Inclusion criteria

We included all original articles on the diagnostic accuracy of EVs in human GBM patients, body fluid isolated EVs, and two types of EVs, including exosomes and microvesicles.

2.2.2. Exclusion criteria

We excluded duplicate citations, non-peer-reviewed, review papers, and book chapters.

2.2.3. Selection of studies

Two reviewers independently (DJ and FJ) evaluated the titles and abstracts of all records to determine whether inclusion and exclusion criteria were met. The inclusion assessment of full papers was conducted by one author (DJ) and checked by a second investigator (AT). In case of disagreement, a consensus was reached by discussion or referral to a third author (YM). We conducted a PRISMA diagram to illustrate the study selection process [20]. 2.2.4. Data collection process

The following items were collected from each article by two investigators (DJ and ER): first author, publication year, country, number of participants (patients and controls), source of EVs, isolation and purification methods, related identified biomarkers, biomarker extraction method, biomarker analyzing/profiling method, area under the curve (AUC), confidence interval 95% (CI95%), true positive (TP), true negative (TN), false positive (FP) and false-negative (FN). The TP, TN, FP, and FN data were extracted from the 2×2 table of studies or (if this table was not provided with studies) calculated using the specificity and sensitivity. The data extracted in a

fully paired method from individual studies. We sought further information from the study authors when necessary. Any disagreements between data collectors were resolved through either discussion or consultation with a third author (AT).

2.3. Assessment of methodological quality and risk of bias

We assessed the methodological quality of the studies independently by two reviewers (DJ and AT) and via the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool, recommended by the Cochrane collaboration for the risk of bias and applicability concerns [23]. This tool assesses the risk of bias by scoring questions in the four domains as follows; 1) Patient Selection (the method of patient selection and the patients included) 2) Index Test (the test being studied and how it was conducted and interpreted) 3) Reference Standard (the reference standard test used and how it was conducted and interpreted) 4) Flow and Timing (the flow of patient inclusion and exclusion, testing procedure and the interval between tests). The first three domains can also assess the applicability concerns regarding the review question. Each of the domains was categorized as high, low, or unclear, and disagreements were resolved by discussion with a third reviewer (RJ).

2.4. Statistical Analysis

We used the metandi and midas modules in the STATA 11.2 (Stata Corporation, College Station, TX, USA) statistical software to perform all the analyses [24,25]. TP, FP, FN, and TN data were used to calculate sensitivity, specificity, positive and negative likelihood ratio, and diagnostic odds ratio. Their pooled estimates and their corresponding CI 95% for exosomes were calculated by using the bivariate and hierarchical meta-analysis that included bivariate mixed-effects regression model and the hierarchical summary ROC (HSROC) modeling [26,27]. Results were displayed graphically on forest-plots and HSROC curve. Heterogeneity between the included

studies was assessed using Cochran's Q test and the inconsistency index (I^2) describing the percentage of total variation across studies due to heterogeneity rather than chance [28]. A p-value ≤ 0.05 and an I^2 value $\geq 50\%$ would indicate substantial heterogeneity. The threshold effect was checked using Spearman's rho, and potential sources of heterogeneity were explored by meta-regression. The sensitivity analysis was performed by omission of outlier studies indicated by Cook's distance, and standardized predicted random effects to investigate the influence of this study on the pooled estimates [25]. We assessed publication bias using Deek's funnel plot and considered a p-value < 0.1 in the Deek's asymmetry test to indicate publication bias [29].

3. Results

3.1. Literature search and study selection

1730 articles (279 from PubMed, 438 from Scopus, 400 from Web of Science, and 613 from Embase) were retrieved, and after duplicate removing, 830 articles were screened, and 802 items, including 259 reviews and 196 conference abstracts, were excluded. Also, the full texts of the 28 remaining records and 19 articles were excluded according to the inclusion and exclusion criteria. Finally, nine eligible studies (from Australia, China, Italy, and the USA) were included in the present systematic review from 2013 to 2019 (Table 1). Figure 1 shows the process of literature search and study selection as a flow diagram based on the PRISMA.

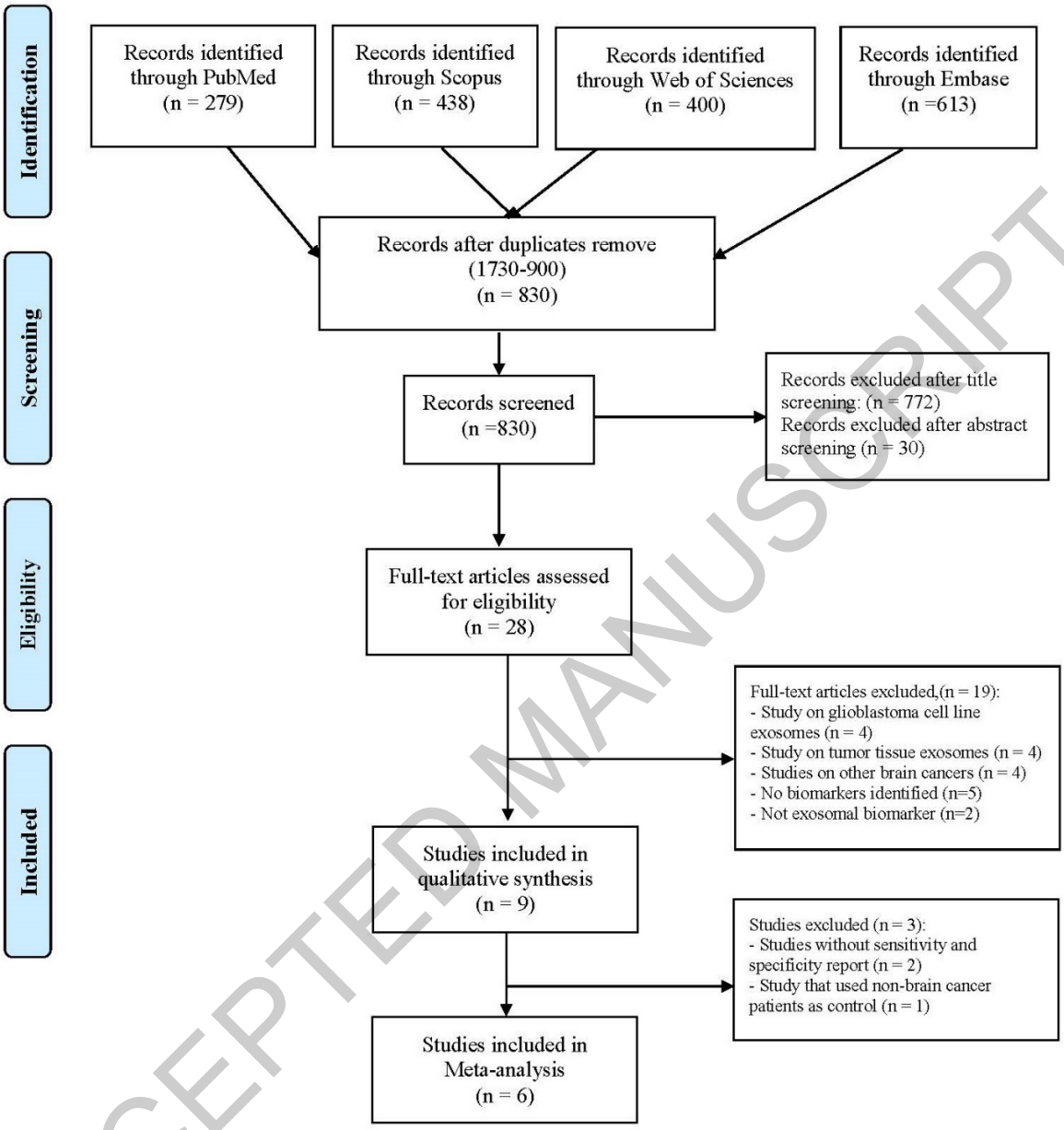

Figure 1 The literature search and study selection process for systematic review according to PRISMA

Table 1 Bibliographic information of included primary studies regarding publication date

Title	Year	Country	Ref.
miR-21 in the Extracellular Vesicles (EVs) of Cerebrospinal Fluid (CSF): A Platform for Glioblastoma Biomarker Development	2013	USA	[30]
A small noncoding RNA signature found in exosomes of GBM patient	2014	China	[31]

serum as a diagnostic tool			
Exosomal levels of miRNA-21 from cerebrospinal fluids associated with poor prognosis and tumor recurrence of glioma patients	2015	China	[32]
A cerebrospinal fluid microRNA signature as biomarker for glioblastoma	2017	USA	[33]
Serum exosomal miR-301a as a potential diagnostic and prognostic biomarker for human glioma	2017	China	[34]
A microRNA signature from serum exosomes of patients with glioma as complementary diagnostic biomarker	2018	Italy	[35]
Deep sequencing of circulating exosomal microRNA allows non-invasive glioblastoma diagnosis	2018	Australia	[36]
Serum long noncoding RNA HOTAIR as a novel diagnostic and prognostic biomarker in glioblastoma multiforme	2018	USA	[37]
Serum miR-29b as a novel biomarker for glioblastoma diagnosis and prognosis	2019	China	[38]

3.2. Quality assessment

According to the results of the QUADAS-2 checklist (Figure S1 and Table S2), in the section of risk of bias, the quality of studies is mostly affected by the reference standard description and the index test. Some studies did not report the diagnostic values for biomarkers. Also, two studies reported the diagnostic accuracy of an exosomal biomarker for GBM from other types of diseases or cancers. In the cases of patient selection, flow, and timing section, six studies are unclear. This is due to undefined patients' pathophysiological condition, sex, and age, as well as some technical analysis and experimental efforts. In the case of applicability, the index test is still suffering from low quality in four studies. However, the rest of the studies have developed their index test compliance with our review question and inclusion criteria.

Only did the study of Manterola et al. [31] perfectly meet our inclusion criteria in all parts of the risk of bias and applicability assessment. Finally, we identified six studies that pass thresholds to be included in the meta-analysis.

3.3. The characteristics of studies included in systematic review

In total, 487 pathologically diagnosed GBM patients were used for EVs derived biomarker discovery in nine included studies. The population of patients ranged from 13-107, and the control population consisted of healthy controls, non-brain tumors [34], and other brain tumor patients [38]. Six studies used the serum, two studies used the CSF, and one study used both serum and CSF as EVs isolation source. Also, one study used CSF from two different anatomical locations (cisterna and lumbar) (Table 2).

As shown in Table 2, EVs isolation was conducted by ultracentrifugation (UC), commercial kits, and size exclusion chromatography. Different kits were used for RNA extraction and the biomarker expression patterns were analyzed via q-RT-PCR and sequencing methods.

Table 2 The methods and workflow data from the included studies for GBM diagnosis.

Patients	HC	Exosomes Source	Exosome Isolation	Biomarker Extraction	Biomarker Analysis	Ref.
13 15	14 16	CSF	UC	mirRCURY kit	qRT-PCR	[30]
10	12	CSF	UC	miRCURY™	qRT-PCR	[33]
12	12	S	SEC	Exosomal RNA Purification Kit	Total RNA chip	[36]
27	43	S	ExoQuick	mirVana kit	qRT-PCR	[34]
25	25	S	Exoquick	Trizol	qRT-PCR	[31]
69	30	S	ExoQuick	Trizol	qRT-PCR	[35]
95	50	CSF and S	UC	RNeasy Kit	RT-qPCR	[32]
43	40	S	Exosome Isolation reagent	mirVana kit	qRT-PCR	[37]
107	80	S	ExoQuick	miRNeasy	qRT-PCR	[38]

M: Male, F: Female, UC: Ultracentrifuge, SEC: Size Exclusion Chromatography, S: Serum, CSF: Cerebrospinal Fluid

3.4 Qualitative synthesis of diagnostic value of EVs RNAs for GBM

Table 3 shows (and Table S3)24 single RNA biomarkers, and three miR-panels. To diagnose GBM in different studies, miR-21, miR-222, miR-29b, miR-320, miR-574-3p, miR-124-3p, RNU6, and HOTAIR biomarkers were individually evaluated. From 24 biomarkers, miR-21 with

85% and 100%, 87% and 93%, 84%, and 77% for sensitivity and specificity, respectively, in three different GBM patient populations has the highest diagnostic accuracy.

Biomarker panels also have high diagnostics accuracy. For instance, a biomarker panel consists of three RNAs has 87% and 86%, 70%, and 71% sensitivity and specificity, respectively, in two groups of GBM patients [31]. Altogether, 24 biomarkers were analyzed for their diagnostic value consist of one long non-coding RNA (LncRNA), one small non-coding RNA (SncRNA), and 22 mi-RNA (Table S4).

Table 3 Diagnostic value data of included primary studies in meta-analysis

Biomarker	**Type of Biomarker**	**AUC**	**SEN (%)**	**SPE (%)**	**PLR**	**NLR**	**DOR**	**Accuracy (%)**	**Ref.**
miR-21*(P1)*	micro-RNA	0.91	85	100	24.64	0.18	133.4	93	[30]
miR-21*(P2)*	micro-RNA		87	88	6.93	0.15	45.5	87	
miR-21, miR-218, miR-193b, miR-331, miR-374a, miR-548c, miR-520f, miR-27b, miR-130b *(P1)*	micro-RNA	0.75	80	67	2.4	0.3	8	73	[33]
miR-21, miR-218, miR-193b, miR-331, miR-374a, miR-548c, miR-520f, miR-27b, miR-130b *(P2)*	micro-RNA	0.83	28	95	5.56	0.76	7.31	63	
miR-574-3p	micro-RNA	0.73	60	60	1.5	0.67	2.25	60	[31]
miR-320	micro-RNA	0.72	64	64	1.78	0.56	3.16	64	
RNU6-1 P1	Small non-coding-RNA	0.85	72	68	2.25	0.41	5.46	70	
RNU6-1 P2	Small non-coding-RNA	0.72	66	67	1.98	0.51	3.88	66	
RNU6, miR-320, miR-574-3p *(P1)*	Small non-coding-RNA & micro-RNA	0.92	88	84	5.5	0.14	38.5	86	
RNU6, miR-320, miR-574-3p *(P2)*	Small non-coding-RNA & micro-RNA	0.77	70	70	2.33	0.43	5.44	70	
miR-21	micro-RNA	0.84	84	77	3.6	0.21	17.37	81	[35]
miR-222	micro-RNA	0.80	57	100	35.13	0.44	79.77	74	
miR-124-3p	micro-RNA	0.78	89	63	2.42	0.18	13.47	78	
miR-21, miR-222, miR-124-3p	micro-RNA	0.87	84	77	3.6	0.21	17.37	81	
HOTAIR	Long non-	0.91	86	88	6.88	0.16	43.17	87	[37]

	coding-RNA								
miR-29b	micro-RNA	0.86	83	81	4.44	0.21	21.43	82	[38]

AUC: Area Under Curve, SEN: Sensitivity, SPE: Specificity, PLR: Positive Likelihood Ratio, NLR: Negative Likelihood Ratio, DOR: Diagnostics Odds ratio

3.5 Meta-analysis

3.5.1 Diagnostic accuracy

We conducted a diagnostic meta-analysis on the data obtained for 16 biomarkers from six studies, including 592 participants (325 GBM patients and 267 healthy controls). We excluded three articles due to the unreported TP, FP, FN, and TN or using the non-healthy controls [32,34,36].

The pooled sensitivity and specificity of biomarkers for detecting the GBM from both serum and CSF were 76% (95% confidence interval [CI] = 68–82%) and 80% (95% CI = 72–86%), respectively. Figure 2 shows a forest plot of the included studies along with sensitivities, specificities, and pooled estimates. A significant heterogeneity was observed in the pooled sensitivity (I^2= 73.44%, P< 0.001) and specificity (I^2= 59.24%, P< 0.001).

The pooled PLR, NLR, and DOR were 3.7 (95% CI: 2.7–5.2), 0.30 (95% CI: 0.23-0.41), and 12 (95% CI: 7–21), respectively (Figure S2 and Figure S3). These statistical measures showed values of I^2> 50% (P< 0.05), suggesting substantial heterogeneity except for PLR.

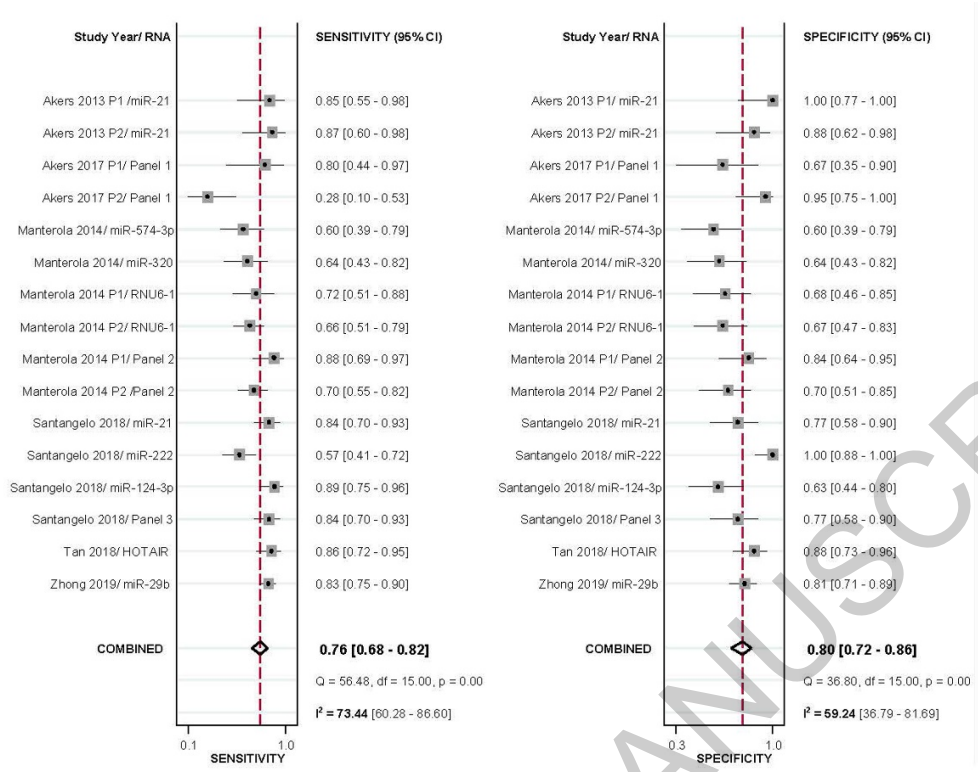

Figure 2. Coupled forest plots show pooled estimates of sensitivity and specificity of biomarkers for GBM diagnosis with corresponding heterogeneity statistics. CI: confidence interval, I^2: inconsistency index.

Figure 3 shows the hierarchical summary receiver operating characteristic (HSROC) curve for diagnostic accuracy of biomarkers. The AUC, as an overall measure for test performance, was 0.85 (95% CI: 0.81-0.87), indicating the high diagnostic accuracy of these biomarkers.

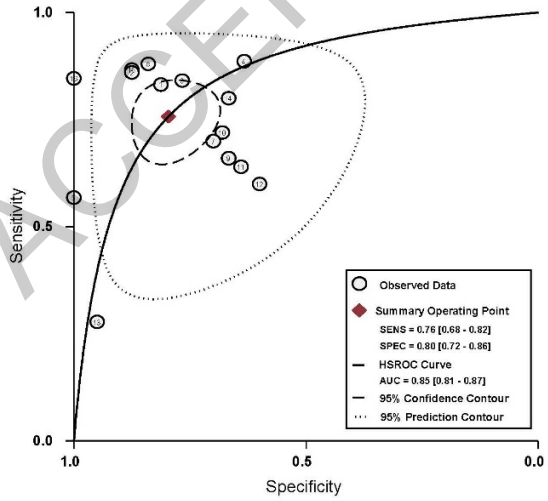

Figure 3. Hierarchical summary receiver operating characteristic (HSROC) curve for EVs derived biomarkers performance in the diagnosis of glioblastoma with confidence and prediction regions around the summary operating point

3.5.2 Publication bias

The potential publication bias was examined by the Deeks' funnel plot. According to Figure S4, a p-value of 0.70 does not show publication bias in the meta-analysis.

3.5.3 Heterogeneity

Due to the lack of threshold effect and the presence of between-study heterogeneity, we conducted a meta-regression to find the source of heterogeneity. The multiple meta-regression of sensitivity and specificity indicated that source and EVs isolation methods, RNA extraction methods, sample size, and the quality of study acted as the potential source of heterogeneity in the pooled estimates (Figure S5). The meta-regression result is shown in Table 4.

3.5.4 Sensitivity analysis

We found two studies, Akers 2017 (p2 [33]) and Santangelo 2018 (miR-222 [35]), as outliers by Cook's distance and standardized predicted random effects (Figure S6). When these outliers were omitted, the pooled sensitivity and specificity were 79% (95% CI 73–84%) and 76% (95% CI 69–81%). Although the omission of these studies did not substantially influence the pooled estimates, it reduced the extent of between-studies heterogeneity in the pooled sensitivity and specificity from I^2 73.44% to 49.25% and 59.24% to 40.68%, respectively (Figure S7).

In addition, the subgroup analysis was performed for serum, and CSF derived EVs biomarkers to find out the superiority of the source. As shown in Table 4, sensitivity is 77% for serum biomarkers and 70% for CSF biomarkers. The difference in sensitivity between these subgroups

is not significant, probably due to the small number of studies. However, the specificity of serum biomarkers is 77% and CSF biomarkers is 89%, which is significant.

Table 4. Results of meta-regression analysis for assessing the source of diagnostic accuracy heterogeneity

Variable	**Category**	**Number of biomarkers**	**Sensitivity % (CI 95%)**	**P-value**	**Specificity % (CI 95%)**	**P-value**
Source of exosomes	Serum	12	0.77 (0.70 - 0.84)	0.59	0.77 (0.69 - 0.84)	0.01
	CSF	4	0.70 (0.52 - 0.88)		0.89 (0.80 - 0.99)	
Isolation method of exosomes	EQ	11	0.76 (0.68 - 0.84)	0.18	0.75 (0.68 - 0.82)	0.001
	Other	5	0.76 (0.61 - 0.90)		0.89 (0.81 - 0.97)	
RNA extraction methods	Trizol	10	0.75 (0.66 - 0.84)	0.06	0.74 (0.66 - 0.82)	0.001
	Other	6	0.77 (0.66 - 0.89)		0.87 (0.80 - 0.94)	
Sample size	>60	8	0.79 (0.71 - 0.87)	0.29	0.80 (0.71 - 0.89)	0.07
	<60	8	0.71 (0.59 - 0.82)		0.79 (0.69 - 0.89)	
Quality of study	High	8	0.73 (0.62 - 0.84)	0.02	0.74 (0.64 - 0.84)	0.001
	Low	8	0.78 (0.69 - 0.87)		0.84 (0.76 - 0.91)	

EQ: Exoquick, **CSF**: Cerebrospinal Fluid.

4 Discussion

Cancer often starts in a single cell with a perturbation in checkpoint molecules by some mutations or other genetic changes [39]. Subsequently, this molecular transformation will appear in whole tumor cells [40]. Identifying and tracking the molecular event of transformation could help develop new therapies and diagnostics [41]. GBM cancer often grows locally and rarely metastasizes to the outside of the CNS. If it is resected in the early stages, it can be completely treated [42], but unfortunately, there are no preoperative routine diagnostic methods for GBM.

In the recent years, circulating molecules (proteins [43], cell-free DNAs [44], and RNAs [45]) and EVs, especially exosomes, were used as diagnostic tools. Recent studies showed that the content of EVs, particularly RNAs (miRNAs, LncRNAs, and mRNAs), could be used for the detection of cancers and the prediction of their stages [46]. For instance, exosomal miR-21 has been widely studied [47-51].

This is the first systematic review and meta-analysis to evaluate EVs diagnostic accuracy for GBM. It aimed to assess how EVs could be used as a non-invasive method for early detection of GBM. We conducted a comprehensive search strategy for searching the databases for all possible EVs-biomarker for GBM. All of the included articles were identified as RNA biomarkers, so we did not find any protein, DNA, lipid, or carbohydrates biomarkers that have met our inclusion and exclusion criteria. It is significant to note that we found some putative protein and DNA biomarkers for GBM diagnosis, whjch were not suitable for this study and needed further experimental assessment.

Different methods of EVs isolation, RNA profiling, and analyzing could affect the results of the test [52-56], we carefully assessed the workflow of studies from the isolation of EVs to the biomarker analysis. Ultracentrifugation (the gold standard for exosome isolation [57]), affinity chromatography method [58], and the exosome isolation kits are used for EVs isolation [59]. As

discussed earlier, EV contents are heterogeneous and may slightly change with different methods of isolation and purification [60]. Methods of downstream analysis of EVs are also the determining factors in the results of diagnostic studies [55,61]. Different RNA isolation kits and two main methods were used for the extraction and profiling of EVs RNAs (q-RT-PCR and Sequencing). According to the results of the extracted data and quality assessment using the QUADAS-2 checklist, we excluded 3 studies and conducted a meta-analysis with six primary research for 16 RNA biomarkers. We calculated pooled estimates of AUC, sensitivity, specificity, DOR, PLR, and NLR for the overall diagnostic value of 16 RNA biomarkers for GBM diagnosis. Our results suggested that the pooled sensitivity and specificity were 76% and 80%, respectively. In addition, the pooled PLR, NLR, and DOR were 3.7, 0.30, and 12, respectively. The PLR suggests that the GBM patients have an about 3.7-fold higher chance for a positive result than a healthy population. Furthermore, 0.30 NLR suggests that from the negative outcome of the index test results, 30% could be GBM positive. Similarly, DOR is an index that correlates with the diagnostic performance of our index test. The higher DOR, the better diagnostic performance [62]. Also, AUC is the most important index to show the overall diagnostic power of an index test. The AUC>0.75 is considered as an acceptable diagnostic performance [63]. According to our results, the value of DOR and AUC were 12 and 0.85, respectively, suggesting that these 16 biomarkers of EVs might serve as a high potential diagnostic tool for GBM. However, significant heterogeneity was observed in the pooled sensitivity, specificity, DOR, and NLR. In the case of publication bias, no evidence for publication bias was found.

Altogether, the multivariate meta-regression of sensitivity and specificity indicated that the source and isolation method, RNA extraction methods, sample size, and quality of study acted as

the potential source of heterogeneity in the pooled estimates. We omitted two studies as outliers and performed a sensitivity analysis. The result of the sensitivity analysis showed that the pooled sensitivity and specificity were 79% and %76.

Since the EVs based diagnosis is a newly-emerged field in biomedical sciences, there are not many studies on every single cancer. Thus the systematic review and meta-analyses studies will be associated with some inherent limitations. The results of the present study and a recent meta-analysis on the assessment of the diagnostic value of exosomes for lung cancer are absolutely consistent. Their results showed that the pooled sensitivity, specificity, PLR, NLR, DOR, and AUC were 82%, 84%, 5.27, 0.21, 25.14, and 0.90, respectively [64]. In addition, a systematic review and meta-analysis of exosomal miR-21 for overall cancer detection showed that the sensitivity and specificity of pooled studies were 75% and 85%, and AUC was 0.93.

Despite our protocols of systematic review and meta-analysis compliance with the latest guidelines for diagnostic studies, our study faced some limitations. The most important limitation was the heterogeneity of biomarkers because we estimated the pooled diagnostic accuracy for 16 different RNA biomarkers. As discussed earlier, this issue is related to the small number of studies on each biomarker. Further studies on these 16 biomarkers could lead to a powerful meta-analysis. The second limitation was the number of included studies and participants, which was likely to be small. More studies with larger numbers of patients and controls could further validate the diagnostic performance of EVs for GBM. The third limitation was that most of the included studies were conducted in China and the USA. Heterogeneity must be taken into account. In this regard, we carried out a meta-regression and sensitivity analysis to detect the potential heterogeneity sources. The workflow, including EVs isolation method, EVs source, RNA profiling method, and sample size, is the source of heterogeneity. In addition, further

studies need to clarify the EVs-derived biomarkers kinetic in the biofluids such as serum and CSF, and the clinical course of GBM for better interpretation of biomarkers expression pattern to a clinical outcome, including response to treatment, relapse, complete treatment, etc.

Ultimately,

Despite the described limitations, our study strongly suggests that the 16 EVs derived RNA biomarkers have high prediction power for GBM. Our study and a few others with meta-analysis showed that EVs derived biomarkers might attract much attention in the near future for the diagnosis of cancers.

5. **Conclusions**

In this study, we found 24 EVs derived RNA biomarkers for GBM diagnosis. In the next step, 16 eligible biomarkers were considered for meta-analysis. Our results suggested that the pooled sensitivity and specificity of these biomarkers were 76% and 80%, respectively. Besides, the pooled PLR, NLR, and DOR were 3.7, 0.30, and 12, respectively. Our data showed the promising potential of these 16 biomarkers for GBM diagnosis.

6. Expert opinion

Unfortunately, most of the discovered biomarkers for GBM have low diagnostic accuracy. New platforms of biomarkers for early diagnosis of this life-threatening cancer is the main goal in the field of biomarker discovery. Despite hundreds of biomarkers identified for brain tumors, the diagnosis and screening of GBM with a biopsy-free and preoperative method remain a big challenge. Regarding the late diagnosis, the survival rate of GBM patients is very low. The very poor prognosis of patients with GBM demands a very rapid development of new platforms of

biomarkers for early diagnosis, screening, response to the treatments, and recurrence through high-quality diagnostic studies.

Here, 24 EVs derived biomarkers were identified for GBM diagnosis by serum or CSF samples, among which RNU6, RNA, miR-21, miR-301 show potential predictive value for GBM. However, these biomarkers still require more studies with large patient populations for a definitive decision. Pooled sensitivity and specificity of 16 biomarkers are 76% and 80%, respectively. Sensitivity is the ability of EVs to identify GBM patients correctly with a 76% probability of positive results. In contrast, the specificity is the ability of EVs to identify healthy persons with an 80% probability that the test will be negative. In addition, PLR, NLR, and DOR for these 16 biomarkers are 3.7, 0.30, and 12, respectively. PLR shows 3.7 times more positive test for GBM patients than healthy persons. In contrast, NLR or the probability of a negative test in GBM is 0.3 times or about one-third of those without the GBM. DOR is the ratio of the odds of a positive test in cases with GBM to the odds of a positive test in the cases without GBM. EVs biomarkers offer odds of a positive test in cases with GBM by 12 times higher than in the cases without GBM. We do not claim that these results offer EVs biomarkers as a definitive diagnostics tool for GBM diagnosis. Since EVs biomarkers have some advantages, we hope that this study will provide a clue for researchers to study more about these potential biomarkers regarding to the criteria described in the next subsection.

6.1 Several recommendations for EVs derived biomarker discovery for GBM

Some criteria and technical aspects should be taken into account by researchers, journals, and relevant regulating agencies for further studies and guidelines for EVs diagnostic research. Given that the EVs research field is one of the newest and active fields, no guidelines have yet been

developed for handling the workflow of EVs for a diagnostic study. The authors of this article have reviewed more than 500 articles regarding the EVs and their development derived biomarkers for the diagnosis of various cancers. Many of these diagnostic studies critically suffer from technical aspects and workflow. Therefore, the authors provide several hints for future studies in EVs biomarker development and analysis. By meeting these suggestions, researchers can improve their study in terms of diagnostic study design and a workflow of research.

Diagnostic accuracy study design. Any research in the field of EVs biomarkers for the diagnosis of GBM or other cancers should meet the protocols and items of STARD for running a diagnostic accuracy study to appropriate sample sizes and other items [65]. Unfortunately, most of the diagnostic accuracy studies, especially in EVs, are missed some of the STARD items, so it is hard to interpret based on their workflow and obtained data.

Technical aspects for EVs-biomarker discovery. Researchers must pay attention to some technical aspects to validate a biomarker. A diagnostic study design following this suggested workflow will make the results of the study easy to interpret for meta-analysis. a) The source of the EVs must be exactly identified. b) The isolated EVs must be well-characterized. Due to the very heterogeneous nature of EVs, there is an argument over the definition of exosome or microvesicle. Therefore, we continuously read some studies that use exosome, EVs, and microvesicles without considering their very similar but different characteristics. In an EVs diagnostic accuracy study, it is highly recommended to use the MISEV2018 guideline [66] for the characterization of EVs populations. c) The isolation method, purity, and concentration of the isolated EVs need to analyze for patients and healthy controls. If applicable, the absence of free

serum or CSF contamination, including proteins and RNAs, should be confirmed. RNA or protein contamination from serum or CSF during the isolation of EVs may affect the results.

Using these recommended workflows, we hope to expedite EVs derived biomarker discovery for GBM.

Availability of Data and Material

Input data for the analyses are available from the corresponding author on request.

Author contributions

D. Jafari was responsible for the conception and design of the study. D.Jafari, A. Tiyuri and E. Rezaei acquired the data. A. Tiyuri, D. Jafari were responsible for analysis and/or interpretation of the data. D. Jafari, A. Tiyuri, R. Jafari, F.J. Shoorijeh and Y. Moradi all drafted the manuscript. D.Jafari, A. Tiyuri, R. Jafari, M. Barati all revised the manuscript in a manner critically for important intellectual content.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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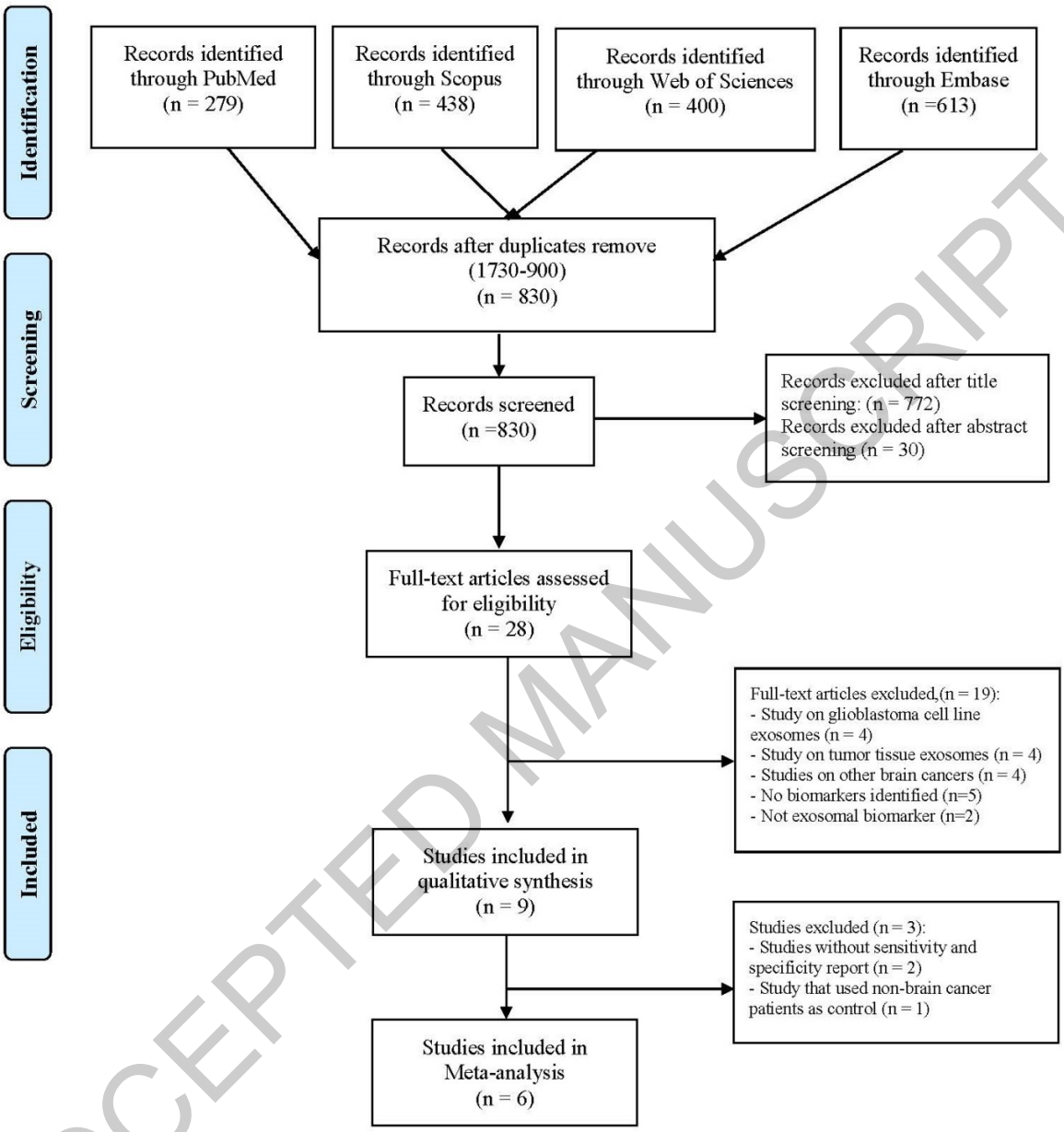

Figure 1

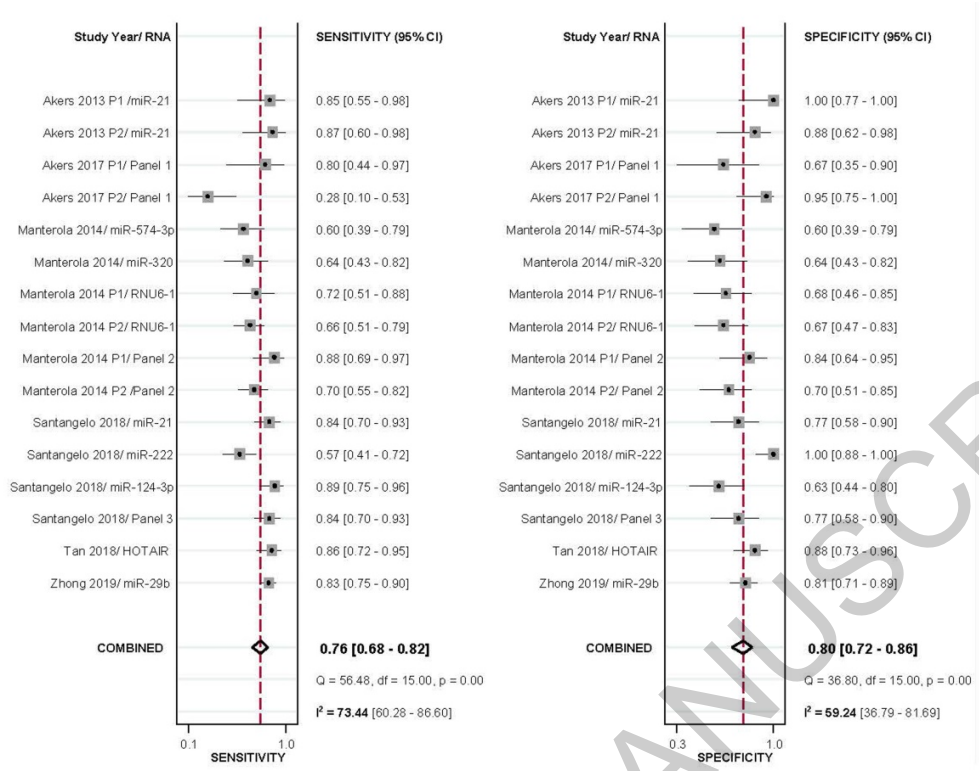

Figure 2

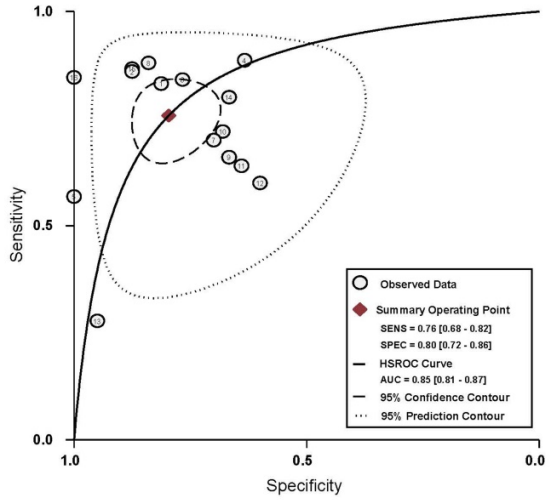

Figure 3

Table S1. Search Strategy of four databases

Database	Record Number	Search Strategy
PubMed	279	("Glioblastoma"[Mesh] OR Glioblastoma[tiab] OR Glioblastomas[tiab] OR ("Grade IV"[tiab] AND Astrocytoma[tiab]) OR ("Grade IV"[tiab] AND Astrocytomas[tiab]) OR GBM[tiab] OR Glyoblastoma[tiab] OR Glyoblastomas[tiab] OR "Malignant Glioma"[tiab] OR "Malignant Gliomas"[tiab] OR Spongioblastoma[tiab] OR Spongioblastomas[tiab] OR "Gliobastoma Multiforme"[tiab]) AND ("Exosomes"[Mesh] OR "Extracellular Vesicles"[Mesh] OR "Cell-Derived Microparticles"[Mesh] OR "Exosomes"[tiab] OR "Exosome"[tiab] OR "Extracellular Vesicles"[tiab] OR "Extracellular Vesicle"[tiab] OR Exosomic[tiab] OR Exosomal[tiab] OR Microvesicles[tiab] OR Microvesicle[tiab] OR "Cell-Derived Microparticles"[tiab] OR "Cell-Derived Microparticle"[tiab] OR Ectosomes[tiab] OR Ectosome[tiab] OR Exovesicles[tiab] OR Exovesicle[tiab] OR (Membrane[tiab] AND Microparticle[tiab]) OR (Membrane[tiab] AND Microparticles[tiab]))
Scopus	438	(TITLE-ABS-KEY(Glioblastoma OR Glioblastomas OR ("Grade IV" AND Astrocytoma) OR ("Grade IV" AND Astrocytomas) OR GBM OR Glyoblastoma OR Glyoblastomas OR "Malignant Glioma" OR "Malignant Gliomas" OR Spongioblastoma OR Spongioblastomas OR "Gliobastoma Multiforme")) AND (TITLE-ABS-KEY("Exosomes" OR "Exosome" OR "Extracellular Vesicles" OR "Extracellular Vesicle" OR Exosomic OR Exosomal OR Microvesicles OR Microvesicle OR "Cell-Derived Microparticles" OR "Cell-Derived Microparticle" OR Ectosomes OR Ectosome OR Exovesicles OR Exovesicle OR (Membrane AND Microparticle) OR (Membrane AND Microparticles)))
Web of Science	400	(TS=(Glioblastoma OR Glioblastomas OR ("Grade IV" AND Astrocytoma) OR ("Grade IV" AND Astrocytomas) OR GBM OR Glyoblastoma OR Glyoblastomas OR "Malignant Glioma" OR "Malignant Gliomas" OR Spongioblastoma OR Spongioblastomas OR "Gliobastoma Multiforme")) AND (TS=("Exosomes" OR "Exosome" OR "Extracellular Vesicles" OR "Extracellular Vesicle" OR Exosomic OR Exosomal OR Microvesicles OR Microvesicle OR "Cell-Derived Microparticles" OR "Cell-Derived Microparticle" OR Ectosomes OR Ectosome OR Exovesicles OR Exovesicle OR (Membrane AND Microparticle) OR (Membrane AND Microparticles)))
Embase	613	('Glioblastoma'/exp OR Glioblastomas:ab,ti OR ('Grade IV':ab,ti AND Astrocytoma:ab,ti) OR ('Grade IV':ab,ti AND Astrocytomas:ab,ti) OR GBM:ab,ti OR Glyoblastoma:ab,ti OR Glyoblastomas:ab,ti OR 'Malignant Glioma':ab,ti OR 'Malignant Gliomas':ab,ti OR Spongioblastoma:ab,ti OR Spongioblastomas:ab,ti OR 'Gliobastoma Multiforme':ab,ti) AND ('Exosome'/exp OR 'Membrane Microparticle'/exp OR Exosomes:ab,ti OR 'Extracellular Vesicles':ab,ti OR 'Extracellular Vesicle':ab,ti OR Exosomic:ab,ti OR Exosomal:ab,ti OR Microvesicles:ab,ti OR Microvesicle:ab,ti OR 'Cell-Derived Microparticles':ab,ti OR 'Cell-Derived Microparticle':ab,ti OR Ectosomes:ab,ti OR Ectosome:ab,ti OR Exovesicles:ab,ti OR Exovesicle:ab,ti OR (Membrane:ab,ti AND Microparticle:ab,ti) OR (Membrane:ab,ti AND Microparticles:ab,ti))

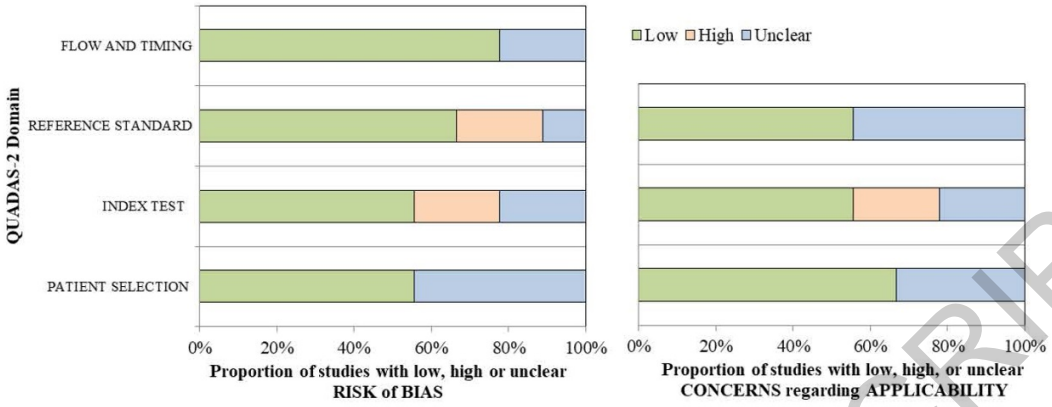

Figure S1. Quality assessment of the included studies using the QUADAS-2 checklist.

Table S2. Tabular presentation for results of quality assessment of included studies with QUADAS-2

STUDY ID	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD
Akers 2013	☺	☺	☺	?	☺	☺	☺
Akers 2017	☺	☺	?	☺	?	☺	?
Ebrahimkhani 2018	☺	?	☺	☺	☺	☹	☺
Lan 2017	?	☹	☺	☺	?	☹	☺
Manterola 2014	☺	☺	☺	☺	☺	☺	☺
Santangelo 2018	?	☺	☹	☺	?	☺	?
Shi 2015	☺	☹	☺	?	☺	?	☺
Tan 2018	?	?	☺	☺	☺	?	?
Zhong 2019	?	☺	☹	☺	☺	☺	?

☺ Low Risk ☹ High Risk ? Unclear Risk

Table S3. Diagnostic value data of included primary studies in meta-analysis

Author (Year)	Biomarker	AUC	95% CI	Tp	Fp	Fn	Tn	SEN	SPE	PLR	NLR	DOR	Accuracy	Cut-off
Akers (2013)	miR-21*(P1)*	0.91	0.797-1	11	0	2	14	0.85	1	24.64	0.18	133.4	0.93	0.25
	miR-21*(P2)*			13	2	2	14	0.87	0.88	6.93	0.15	45.5	0.87	(Copy/EV)
Akers (2017)	miR-21, miR-218, miR-193b, miR-331, miR-374a, miR-548c, miR-520f, miR-27b, miR-130b *(P1)*	0.75	0.53-0.97	8	4	2	8	0.8	0.67	2.4	0.3	8	0.73	0.4 (FC)
	miR-21, miR-218, miR-193b, miR-331, miR-374a, miR-548c, miR-520f, miR-27b, miR-130b *(P2)*	0.83	0.63-0.96	5	1	13	19	0.28	0.95	5.56	0.76	7.31	0.63	0.4 (FC)
Manterola (2014)	miR-574-3p	0.73	0.58–0.89	15	10	10	15	0.6	0.6	1.5	0.67	2.25	0.60	0.454 (FC)
	miR-320	0.72	0.56–0.87	16	9	9	16	0.64	0.64	1.78	0.56	3.16	0.64	0.477 (FC)
	RNU6-1 P1	0.85	0.74–0.96	18	8	7	17	0.72	0.68	2.25	0.41	5.46	0.70	0.454 (FC)
	RNU6-1 P2	0.72	0.60–0.84	33	10	17	20	0.66	0.67	1.98	0.51	3.88	0.66	0.372 (FC)
	RNU6, miR-320, miR-574-3p *(P1)*	0.92	0.84–1	22	4	3	21	0.88	0.84	5.5	0.14	38.5	0.86	0.349 (FC)
	RNU6, miR-320, miR-574-3p *(P2)*	0.77	0.65-0.90	35	9	15	21	0.7	0.7	2.33	0.43	5.44	0.70	0.347 (FC)
Santangelo (2018)	miR-21	0.84	0.75–0.93	37	7	7	23	0.84	0.77	3.6	0.21	17.37	0.81	1.78 (FC)
	miR-222	0.80	0.69–0.89	25	0	19	30	0.57	1	35.13	0.44	79.77	0.74	129.41 (FC)
	miR-124-3p	0.78	0.67–0.89	39	11	5	19	0.89	0.63	2.42	0.18	13.47	0.78	2.95 (FC)
	miR-21, miR-222, miR-124-3p	0.87	0.78–0.95	37	7	7	23	0.84	0.77	3.6	0.21	17.37	0.81	0.90 (FC)
Tan (2018)	HOTAIR	0.91	NR	37	5	6	35	0.86	0.88	6.88	0.16	43.17	0.87	10.8 (FC)
Zhong (2019)	miR-29b	0.86	NR	89	15	18	65	0.83	0.81	4.44	0.21	21.43	0.82	NR

FC: Fold Change, AUC: Area Under Curve, SEN: Sensitivity, SPE: Specificity, TN: True Positive, TN: True Negative, FP: False Positive, FN: False Negative, PLR: Positive Likelihood Ratio, NLR: Negative Liklihood Ratio, DOR: Diagnostics Odds ratio, NR: Not Reported

Table S4. The exosomal biomarkers for GBM diagnosis in the included nine studies

No	Biomarker	Type	First Author
1	miR-21	micro-RNA	Akers (2013), Akers (2017), Santangelo, Shi.
2	miR-486-5p	micro-RNA	Ebrahimkhani
3	miR-182-5p	micro-RNA	
4	miR-328-3p	micro-RNA	
5	miR-339-5p	micro-RNA	
6	miR-340-5p	micro-RNA	
7	miR-543	micro-RNA	
8	miR-485-3p	micro-RNA	
9	miR-301a	micro-RNA	Lan
10	RNU6-1	Small non-coding-RNA	Manterola
11	miR-320	micro-RNA	
12	miR-574-3p	micro-RNA	
13	miR- 222	micro-RNA	Santangelo
14	miR-124-3p	micro-RNA	
15	lncRNA HOTAIR	Long non-coding-RNA	Tan
16	miR-193b	micro-RNA	Akeres
17	miR-218	micro-RNA	
18	miR-331	micro-RNA	
19	miR-374a	micro-RNA	
20	miR-27b	micro-RNA	
21	miR-130b	micro-RNA	
22	miR-520f	micro-RNA	
23	miR-548c	micro-RNA	
24	miR-29b	micro-RNA	Zhong

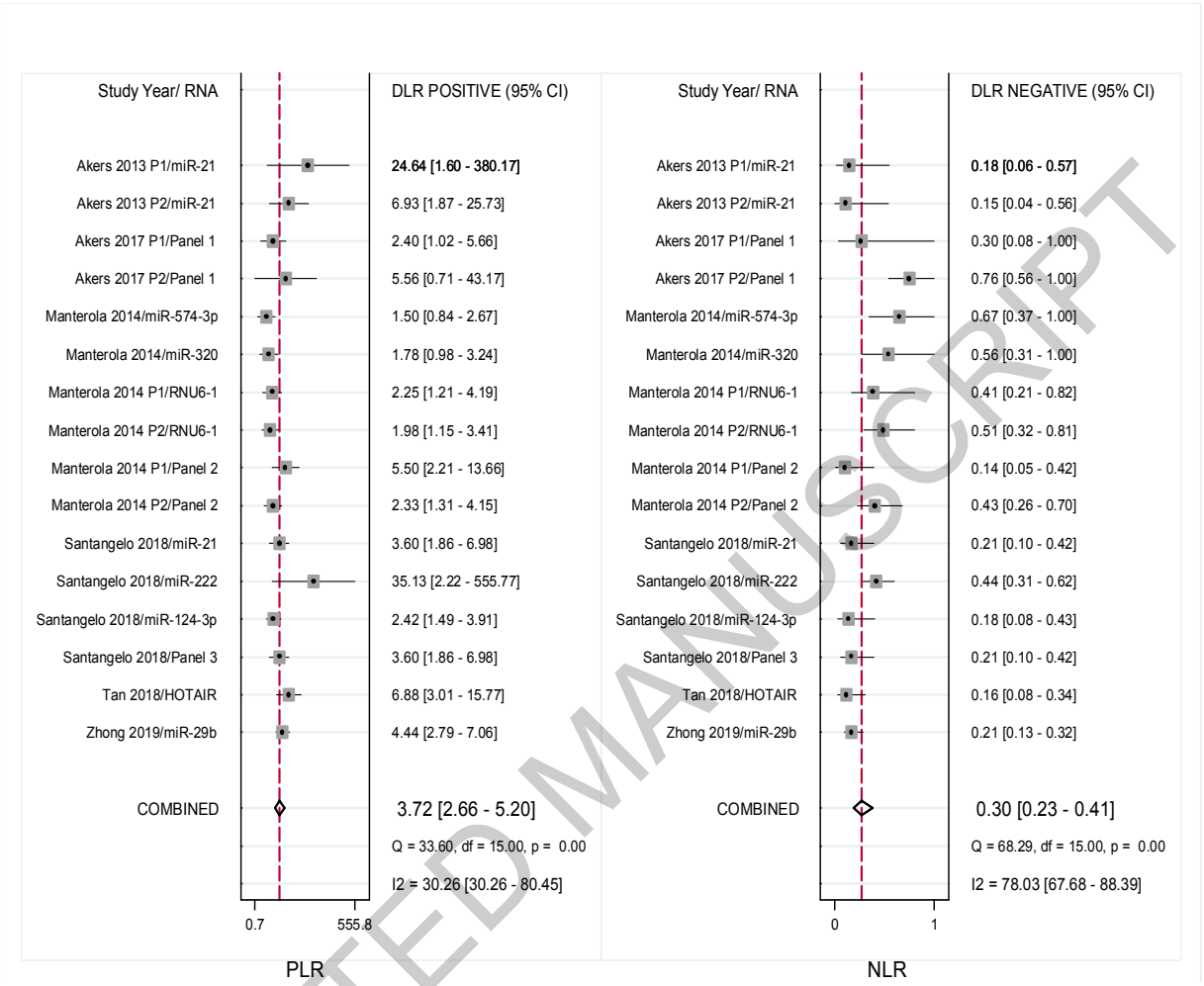

Figure S2. Coupled forest plots show pooled estimates of positive and negative likelihood ratios (PRL & NLR) of exosomal biomarkers for glioblastoma diagnosis with corresponding heterogeneity statistics. CI: confidence interval, I^2: inconsistency index.

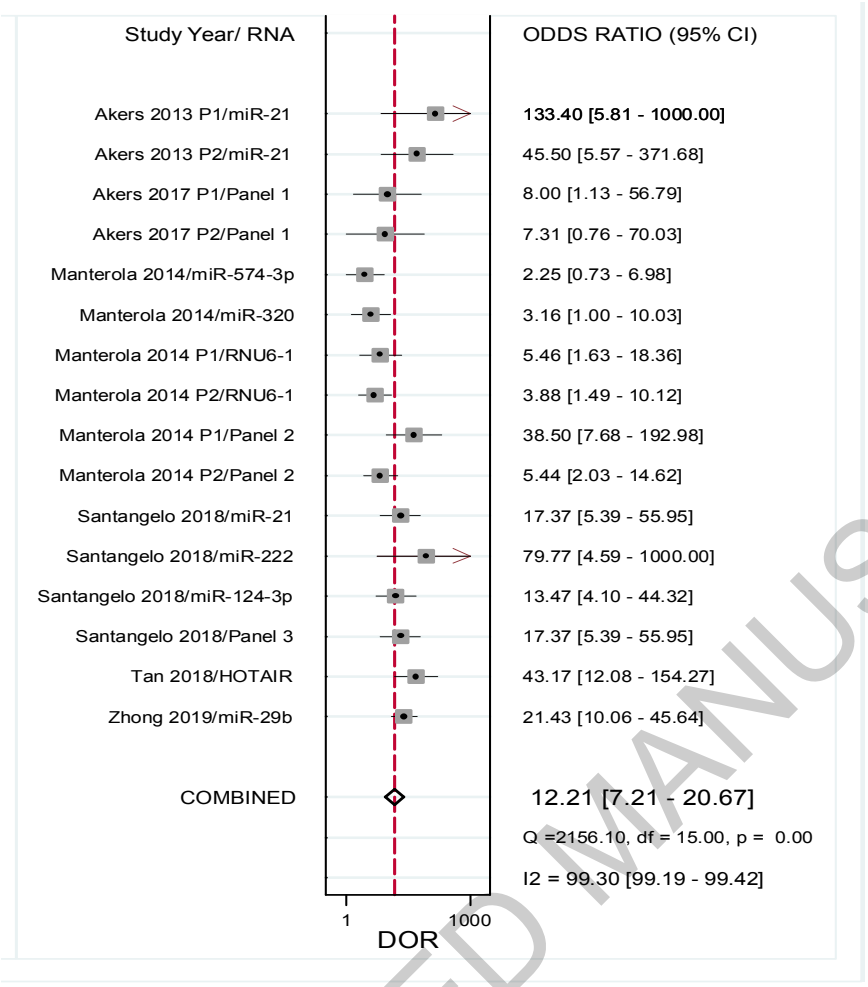

Figure S3. Forest plot shows pooled estimate of diagnostic odds ratio (DOR) of exosomal biomarkers for glioblastoma diagnosis with corresponding heterogeneity statistics. CI: confidence interval, I^2: inconsistency index.

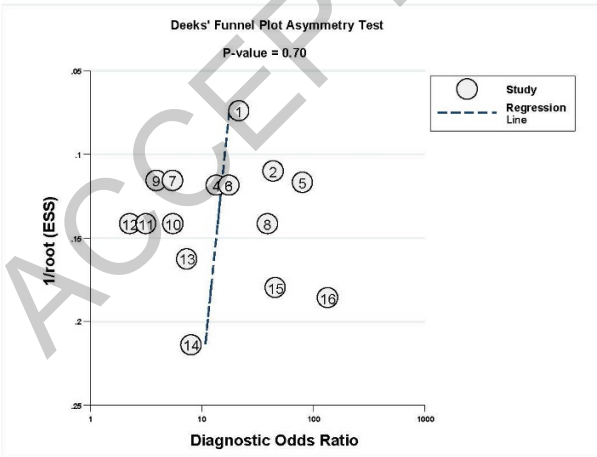

Figure S4. Deek's funnel plot and asymmetry test for evaluating potential publication bias. ESS: the effective sample size.

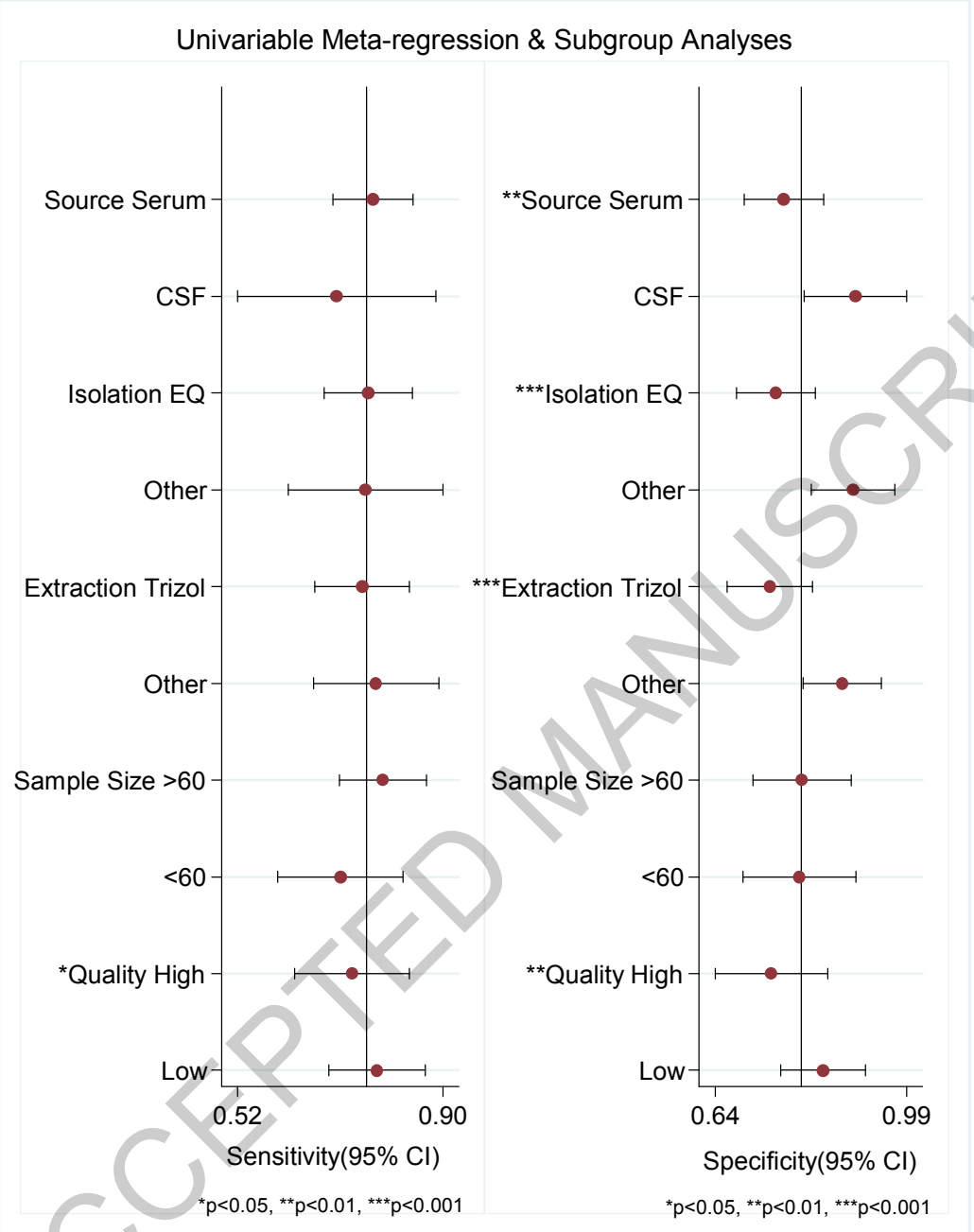

Figure S5. Coupled forest plots show result of multiple meta-regression and subgroup analysis on pooled sensitivity and specificity.

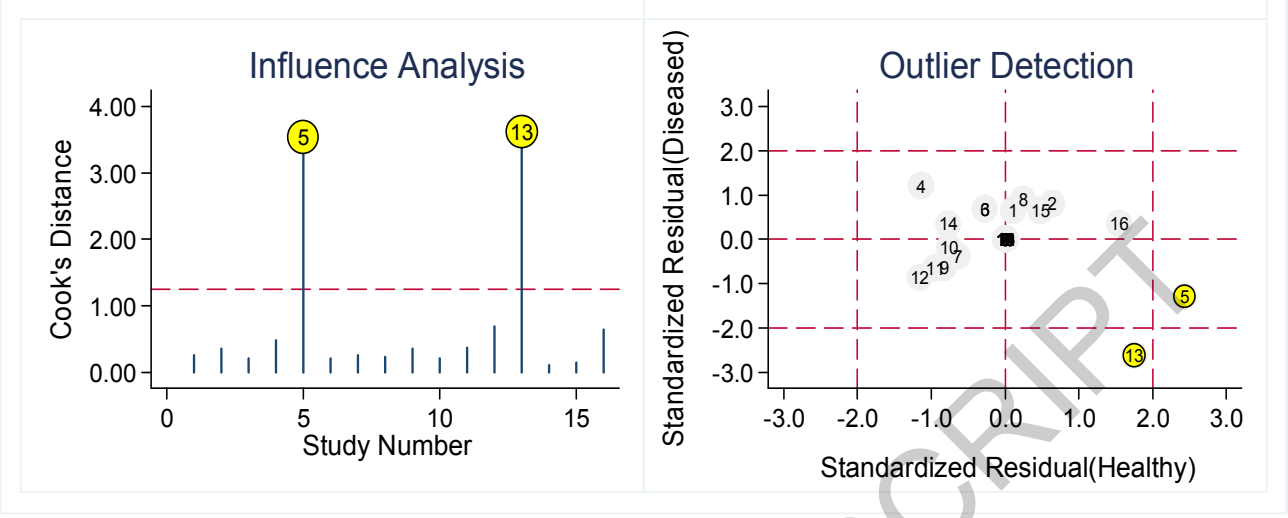

Figure S6. Graphical depiction for outlier detection analysis using Cook's distance and standardized predicted random effects.

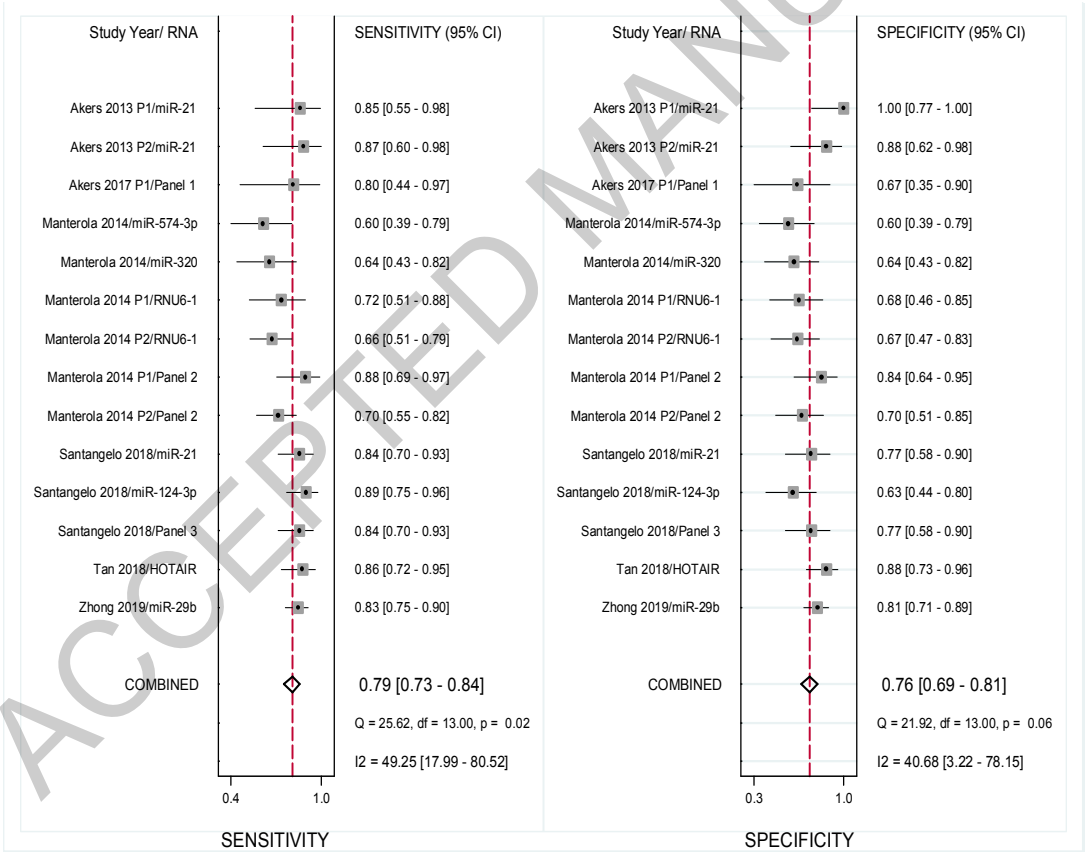

Figure S7. Coupled forest plots show pooled estimates of sensitivity and specificity and corresponding heterogeneity statistics after outlier omission in sensitivity analysis. CI: confidence interval, I^2: inconsistency index.