Blood-based circulating microRNAs as diagnostic biomarkers for subclinical carotid atherosclerosis: A systematic review and meta-analysis with bioinformatics analysis

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ABSTRACT

Background: Atherosclerosis in carotid arteries can remain clinically undetected in its early development until an acute cerebrovascular event such as stroke emerges. Recently, microRNAs (miRNAs) circulating in blood have emerged as potential diagnostic biomarkers, but their performance in detecting subclinical carotid atherosclerosis has yet to be systematically researched.
Aim: To investigate the diagnostic performance of circulating miRNAs in detecting subclinical carotid atherosclerosis.
Methods: We systematically searched five electronic databases from inception to July 23, 2022. Subclinical carotid atherosclerosis was defined using carotid intima-media thickness (CIMT). Diagnostic accuracy parameters and correlation coefficients were pooled. A gene network visualisation and enrichment bioinformatics analysis were additionally conducted to search for potential target genes and pathway regulations of the miRNAs.
Results: Fifteen studies (15 unique miRNAs) comprising 2542 subjects were identified. Circulating miRNAs had a pooled sensitivity of 85% (95% CI 80%–89%), specificity of 84% (95% CI 78%–88%), positive likelihood ratio of 5.19 (95% CI 3.97–6.80), negative likelihood ratio of 0.18 (95% CI 0.13–0.23), diagnostic odds ratio of 29.48 (95% CI 21.15–41.11), and area under the summary receiver operating characteristic curve of 0.91 (95% CI 0.88–0.93), with a strong correlation to CIMT (pooled coefficient 0.701; 95% CI 0.664–0.731). Bioinformatics analysis revealed a major role of the miRNAs, as shown by their relation with *CCND1*, *KCTD15*, *SPARC*, *WWTR1*, *VEGFA* genes, and multiple pathways involved in the pathogenesis of carotid atherosclerosis.
Conclusion: Circulating miRNAs had excellent accuracy in detecting subclinical carotid atherosclerosis, suggesting their utilisation as novel diagnostic tools.

1. Introduction

Atherosclerosis is characterised by the formation of plaques in the arteries, which restricts blood flow. Once the plaque ruptures, there will be the accumulation of cells, cholesterol, lipid deposition and extracellular matrix, leading to vessel obstruction that may be threatening [1].

Like all such processes, atherosclerosis is a chronic and progressive inflammatory disease that exists along a continuum from subclinical to patent clinical atherosclerotic vascular disease. It can start early in life and remain clinically undetected until an acute event such as myocardial infarction or stroke [2]. Subclinical atherosclerosis is an early indicator of atherosclerotic burden, and early recognition can slow or prevent its progression to overt cardiovascular diseases [3]. Thus, individuals with subclinical atherosclerosis are a vital priority for primary prevention. However, it has also become a challenge for primary care providers to identify this condition.

In diagnosing atherosclerosis, mainly in carotid arteries, carotid intima-media thickness (CIMT) is suggested to be an important biomarker. CIMT is measured in B-mode ultrasound images of the carotid tree as a typical double line of the arterial wall [4]. Conventional methods, which are B-mode ultrasound and doppler mode, have been used to identify, discover, and indicate an atherosclerotic plaque. However, they have many limitations in precision, the profundity of scanning, and obtaining adequate ultrasound windows for the superficial vessels and the plaque status [5].

The prevalence of carotid atherosclerosis detected using CIMT measurement is still high, even increasing every year. A study by Song et al. [6] estimated about 677.32 million people had increased CIMT in 2000. This number increased to 1.06 billion in 2020. The number of carotid plaque presence is also growing, from 513.16 to 815.76 million between 2000 and 2020. Considering its future adverse consequences, high prevalence, and limitations in its detection using currently available methods, early and accurate identification of individuals with carotid atherosclerosis using newer and more practical approaches has become crucial, especially in its subclinical stage.

MicroRNAs (miRNAs) are the most abundant class of short single-strand non-coding RNAs that play key roles in many biological processes, such as autophagy, cell apoptosis, proliferation, and differentiation. MiRNAs are also involved in regulating the pathogenesis of cardiovascular diseases, such as atherosclerosis, ranging from its risk factors to plaque initiation, progression, and atherosclerotic plaque rupture [7]. Another study also mentioned that changes in miRNAs could be detected in different cardiovascular diseases, and their expression could be a biomarker for early diagnosis of cardiovascular diseases such as atherosclerosis [8]. Interestingly, a recent study found that miRNAs are highly expressed in the vasculature, and their expression is downregulated in diseased vessels as in the blood serum and plasma [9]. Therefore, miRNAs circulating in serum and plasma could serve as promising non-invasive biomarkers for asymptomatic atherosclerotic disease. In addition to frequent and widespread use, blood samples are highly accessible, making miRNA measurement in serum and plasma convenient. Moreover, miRNAs are easier to detect with current technology, such as quantitative real-time polymerase chain reaction (qRT-PCR) [10].

Although several studies have shown that miRNAs are potential biomarkers for subclinical carotid atherosclerosis with high diagnostic accuracy, no study has concluded the outcomes. Moreover, there has not been a meta-analysis and bioinformatics analysis made that investigated miRNAs and subclinical carotid atherosclerosis. Therefore, in this study, we aim to explore the diagnostic accuracy and the role of circulating miRNAs as emerging biomarkers of subclinical carotid atherosclerosis. We hypothesised that circulating miRNAs in blood have significant diagnostic power in detecting subclinical carotid atherosclerosis.

2. Methods

This systematic review and meta-analysis were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines [11] (see Supplementary material, Table S1 for the completed PRISMA 2020 checklist of this study) and guided by the Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy Version 2.0 [12]. The protocol of this study has been registered on the International Prospective Register of Systematic Reviews (PROSPERO) (https://www.crd.york.ac.uk/prospero/) with the registration number CRD42022352427.

2.1. Search strategy

A computerised systematic literature data search was conducted in PubMed, Scopus, Web of Science, ProQuest, and Cumulative Index to Nursing and Allied Health Literature (CINAHL) via EBSCO for studies published up to July 23, 2022. We additionally performed a manual hand-search on Google and the reference lists of the included studies to maximise the search results. The following main keywords were initially established: "microRNAs", "atherosclerosis", and "carotid arteries". We subsequently added several Medical Subject Headings (MeSH) and other free-text terms to construct database-specific search terms. The full search strings for each database are provided in Table S2. No publication date and language restrictions were set in all searches.

2.2. Selection of studies

Search results from each database were pooled and managed collectively using Google Sheets (https://docs.google.com/spreadsheets/) (Google LLC, Mountain View, CA, USA). After deduplicating, the remaining articles were screened based on their title and abstract. All articles included in the subsequent screening step were sought for retrieval. Afterwards, studies were thoroughly assessed according to the pre-specified eligibility criteria. The reasoning for the exclusion of each article on each screening step was declared as appropriate in the spreadsheet. The literature searches and overall study selection process were performed by BSW, VV, FMA, MSSA, and MVA. Any disagreements were reconciled through a group discussion.

2.3. Eligibility criteria

We applied the Population, Index Test, Comparator, Outcome (PICO) framework [13] (Table S3) designed for systematic reviews of diagnostic test accuracy studies as the basis for formulating the eligibility criteria. To be included in the systematic review and meta-analysis, studies had to meet the following criteria: (1) the study population consisted of adult subjects aged 18 years or older; (2) defined subclinical or asymptomatic carotid atherosclerosis using CIMT; (3) investigated the diagnostic accuracy of circulating miRNAs in blood serum or plasma for subclinical carotid atherosclerosis; and (4) employed an observational design (case-control, cross-sectional, or cohort studies). We accepted studies published in any language. Studies were excluded if: (1) the study was a review article, case report, case series, or conference abstract; or (2) the full-text was irretrievable.

2.4. Data extraction and quality assessment

Three investigators (FMA, MSSA, and MVA) performed data extraction from each included study using a pre-specified checklist designed and tabulated within the spreadsheet by BSW and VV. Afterwards, the collected data were checked for their eligibility by two investigators (BSW and VV) and any disagreements were promptly resolved. The data extracted include the name of the first author and year of publication, study location (country and region), study design, diagnostic criteria of subclinical carotid atherosclerosis, specimen and assay method used for detecting the circulating miRNAs, sample size, sex (% of females), age, type and regulation mode of miRNAs, correlation strength of miRNAs with CIMT, and diagnostic accuracy parameters of circulating miRNAs (area under the curve [AUC], cut-off, sensitivity, and specificity). The extracted characteristics and outcomes of each included study were then presented qualitatively in a tabular format.

The methodological quality assessment of the included studies was conducted by two independent reviewers (BSW and VV) using the

Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool [14]. Discordance in judgements was resolved simultaneously in a consensus with a third reviewer (APW). The QUADAS-2 tool consists of four domains (patient selection, index test, reference standard, and flow and timing) assessed in terms of risk of bias and concerns regarding applicability using specific signalling questions. We further tailored the signalling questions of the risk of bias assessment to have greater relevance to the current study. Signalling questions from studies by McCrea et al. [15], Meursinge Reynders et al. [16], and Wade et al. [17] were sampled as the basis for formulating additional question items (see Table S4 for full details). Reviewers were required to respond "yes", "no", or "unclear" to each of the signalling questions. The risk of bias and concerns of applicability in each domain were then rated as "low", "high", or "unclear" based on the responses and further visualised using Review Manager version 5.4 (The Cochrane Collaboration, The Nordic Cochrane Centre, Copenhagen, Denmark). A study was judged to have a moderate overall risk of bias or applicability concern when there was exactly one risk or concern domain rated as "unclear". A high overall bias risk or concern regarding applicability was considered when the study had a high-rated risk or concern in at least one domain or an unclear-rated risk or concern in multiple domains. Otherwise, studies were judged as having a low risk of bias or applicability concern.

2.5. Statistical analysis

2.5.1. Meta-analysis

All statistical analyses were performed using STATA version 16.0 (Stata Corporation, College Station, TX, USA). For the primary outcome, we conducted diagnostic test accuracy meta-analyses on circulating miRNAs in detecting subclinical carotid atherosclerosis. We additionally performed a secondary meta-analysis to estimate the pooled correlation coefficient between circulating miRNAs expression and CIMT values. Heterogeneity was evaluated using the Cochran's Q statistic and quantified using the Higgins' I^2 statistic. To demonstrate the level of heterogeneity, we considered I^2 values of 0%, 25%, 50%, and 75% as negligible, low, moderate, and high heterogeneity, respectively. A p-value of <0.05 was used to indicate statistical significance in all analyses.

Diagnostic accuracy meta-analyses were performed using a bivariate model to estimate the pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR), and area under the summary receiver operating characteristic (AUSROC) curve along with their corresponding 95% confidence intervals (CIs). The AUSROC curve value was then interpreted as similar to the AUC, where 0.5 indicates that circulating miRNAs have no ability to distinguish patients with and without subclinical carotid atherosclerosis, while 0.7 to 0.8 is considered an acceptable diagnostic power, 0.8 to 0.9 is considered excellent, and more than 0.9 suggests an outstanding discriminatory power [18]. The correlation between the sensitivity and 1 – specificity of circulating miRNAs between studies was explored using the Spearman's correlation analysis to assess the diagnostic threshold effect. The threshold effect is one of the major causes of heterogeneity in a diagnostic accuracy meta-analysis, which arises due to variability in the cut-off values used between studies. A positive Spearman's correlation coefficient with $p < 0.05$ suggests a significant diagnostic threshold effect.

The pooled correlation coefficient meta-analysis between circulating miRNAs and CIMT was conducted using the Hedges-Olkin method under a DerSimonian-Laird random-effects model. The Hedges-Olkin method is based on a meta-analysis with a Fisher's Z-transformation. We first transformed the absolute value of the correlation coefficient obtained from each study into a Fisher's Z-value. Standard errors were then calculated from the total sample size. Afterwards, the pooled Z-transformation value and its 95% CI were back transformed into a pooled correlation coefficient and its corresponding 95% CI. The strength of the pooled correlation was considered as follows: 0.00–0.19 as very weak,

0.20–0.39 as weak, 0.40–0.59 as moderate, 0.60–0.79 as strong, and 0.80–1.00 as very strong correlation [19].

2.5.2. Analysis of publication bias

Publication bias for the meta-analysis of diagnostic accuracy was evaluated using the Deeks' funnel plot, while publication bias for the meta-analysis of correlation coefficients was assessed visually using the inverted funnel plot and quantitatively using the Egger's test. Analyses were conducted to detect small study effects and other potential reporting biases.

2.5.3. Sensitivity analysis

Sensitivity analyses for all outcomes were conducted in three methods by excluding: (1) each study individually (leave-one-out analysis); (2) studies detected as outliers; and (3) moderate and high risk of bias studies. After each analysis, the consistency and significance of the meta-analysis results were re-evaluated. In the meta-analysis of diagnostic accuracy, a study was considered an outlier if the study's data point was located outside the outer oval region of the bivariate boxplot. This bivariate boxplot describes the degree of interdependence between the sensitivity and specificity from each study, where the inner and outer ovals represent the median distribution and 95% CIs of all the data points in the analysis, respectively. Outliers in the meta-analysis of correlation coefficients were identified by visually examining the forest plot. A study with a 95% CI located outside the 95% CI of the pooled result was considered an outlier. Based on our study protocol, we stated that sensitivity analyses would also be conducted by excluding studies with a small sample size ($n < 100$). However, this approach was not possible since none of our included studies had a sample size of <100.

2.5.4. Subgroup and meta-regression analyses

We prioritised performing subgroup and meta-regression analyses for the primary outcome to search for potential causes of heterogeneity. We conducted subgroup analyses based on: (1) study design; (2) specimen of miRNAs; and (3) regulation mode of miRNAs. Meta-regression analyses were carried out for: (1) year of publication; (2) % of females; (3) sample size; and (4) mean population age. As stated in our protocol, we would perform a subgroup analysis based on the study location. Nevertheless, this approach was also not possible as all of the included studies were conducted in China.

2.5.5. Bioinformatics analysis

Bioinformatics analyses were conducted on the 15 identified miRNAs from our included studies using miRTargetLink 2.0 (https://www.ccb.uni-saarland.de/mirtargetlink2/) [20] to provide a better understanding of their functional role. These miRNAs with their available complementary structures (5p and 3p strands) were then included in the unidirectional search field on miRTargetLink 2.0 to obtain information on miRNA interactions through a network visualisation of the target genes for each miRNA in humans. Additionally, we applied miRNA enrichment analysis and annotation tool (miEAA) 2.0 to identify diseases and biological processes significantly regulated by the identified miRNAs [21]. The related-disease pathways were explored from several databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Gene Ontology (GO) annotations, and Mammal ncRNA-Disease Repository (MNDR).

3. Results

3.1. Overview of study selection

A PRISMA flowchart of the study selection process is depicted in Fig. 1. Initial searches in the five databases resulted in a total of 941 hits. We identified duplicates, and 509 records were subsequently removed. Of the remaining 432 records, 204 and 146 were excluded based on their title and abstract, respectively. Six conference abstracts and two articles
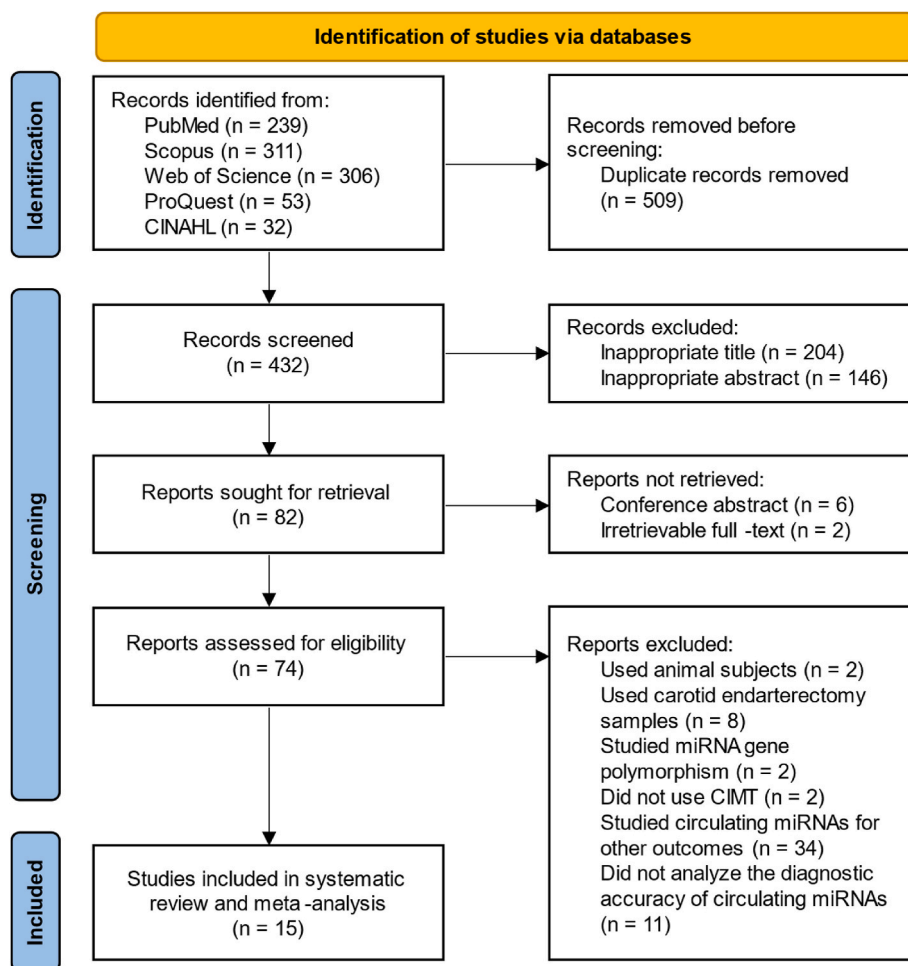
Fig. 1. PRISMA flowchart of the study selection process. **CIMT**, carotid intima-media thickness; **CINAHL**, Cumulative Index to Nursing and Allied Health Literature; **miRNA**, micro-ribonucleic acid; **PRISMA**, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

with no available full-text were not retrieved. We thoroughly reviewed the remaining 74 studies and further excluded 59 studies due to the following: inappropriate population with animals as study subjects (n = 2) and carotid endarterectomy as samples (n = 8); studies on miRNA gene polymorphism (n = 2); not using CIMT in defining subclinical carotid atherosclerosis (n = 2); irrelevant disease target (n = 34); and not investigating the accuracy of circulating miRNAs (n = 11). Ultimately, the entire screening process led to the inclusion of 15 eligible studies [22–36] in this systematic review.

3.2. Characteristics and outcomes of included studies

The characteristics of the included studies are summarised in Table 1. The total sample accumulated was 2542 adults (mean age: 48.0–64.5 years), from which 1403 diagnosed with subclinical carotid atherosclerosis and 1139 constituted the control samples. The total number of females from all studies was 1,214, which accounted for 47.8% of the total study population. All studies were located in China. Four studies used a cross-sectional design with blood plasma as the specimen for miRNAs, while the rest were case-control studies with blood serum as the specimen (n = 11). The CIMT cut-off values used to define subclinical carotid atherosclerosis varied between studies.

The outcomes of the 15 included studies, each with a unique type of circulating miRNA, are provided in Table S5. Eleven miRNAs (miR-18a-5p, miR-29a, miR-29b, miR-29c, miR-146a, miR-183-5p, miR-186-5p, miR-192-5p, miR-374, miR-488, and miR-675-3p) were upregulated in their expression in patients with subclinical carotid atherosclerosis,

while four miRNAs (miR-199a-3p, miR-211-5p, miR-532-5p, and miR-637) were downregulated. All studies reported a significant correlation between circulating miRNAs and CIMT. The diagnostic accuracy of circulating miRNAs was variable between studies, with miR-675-3p as the highest and miR-29a as the lowest.

3.3. Quality assessment of included studies

The overall risk of bias and concerns regarding the applicability of each study according to the QUADAS-2 tool is provided in Table 1, while the domain-specific details of the results are shown in Fig. S1. In terms of risk of bias, eight studies were rated low in all domains, thus having a low overall risk of bias. Six studies were judged as having a moderate bias risk since there was no clear information on whether the study population was selected in a consecutive or random manner concerning the patient selection domain. One study by Xu et al. [26] was considered to have a high bias risk as there were no sufficient details for judging multiple signalling questions in the patient selection domain. Consequently, this study was regarded as having a moderate concern of applicability due to the unclear judgement in the patient selection domain. The other fourteen studies were rated low in terms of overall applicability concerns.

3.4. Correlation between circulating miRNAs and CIMT

3.4.1. Meta-analysis and publication bias

Fifteen studies with a total sample of 2542 (1403 patients and 1139

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Table 1
Characteristics and quality of the included studies.

First Author, Year	Study Location	Study Design	Subclinical Carotid Atherosclerosis Diagnostic Criteria	Specimen	Assay Method	% F	Sample Size		Age[a]		MiRNA Type	Regulation Mode	QUADAS-2	
							Patients (F)	Control (F)	Patients	Control			Risk of Bias	Applicability Concerns
Guo et al., 2020 [22]	China, Asia	Cross-sectional	Common carotid IMT ≥1.0 mm or carotid bifurcation IMT ≥1.2 mm	Plasma	qRT-PCR	34	48 (N/A)	52 (N/A)	63.83 ± 10.84	51.10 ± 10.22	miR-146a	Upregulated	Low	Low
Huang et al., 2016 [23]	China, Asia	Cross-sectional	CIMT ≥0.9 mm	Plasma	qRT-PCR	48.82	85 (44)	85 (39)	50.48 ± 5.78	51.22 ± 5.42	miR-29a	Upregulated	Low	Low
Huang et al., 2017 [29]	China, Asia	Cross-sectional	CIMT ≥0.9 mm	Plasma	qRT-PCR	48.82	85 (44)	85 (39)	50.48 ± 5.78	51.22 ± 5.42	miR-29b	Upregulated	Low	Low
Huang et al., 2018 [30]	China, Asia	Cross-sectional	CIMT ≥0.9 mm	Plasma	qRT-PCR	48.82	85 (44)	85 (39)	50.48 ± 5.78	51.22 ± 5.42	miR-29c	Upregulated	Low	Low
Li et al., 2021 [31]	China, Asia	Case-control	CIMT ≥0.9 mm and ≤1.2 mm	Serum	qRT-PCR	52.43	125 (61)	60 (36)	50.71 ± 6.06	49.62 ± 4.73	miR-488	Upregulated	Low	Low
Sun B et al., 2020 [32]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	47.83	104 (48)	80 (40)	48.89 ± 5.06	47.98 ± 5.53	miR-186-5p	Upregulated	Moderate	Low
Sun B et al., 2021 [33]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	47.22	108 (52)	72 (33)	51.62 ± 5.54	51.22 ± 5.58	miR-183-5p	Upregulated	Moderate	Low
Sun H et al., 2020 [34]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	48.33	77 (38)	103 (49)	48.00 (43.00)	49.15 (45.00)	miR-532-5p	Downregulated	Moderate	Low
Sun X et al., 2021 [35]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	46	100 (43)	50 (26)	49.94 ± 5.48	50.70 ± 5.78	miR-199a-3p	Downregulated	Low	Low
Teng et al., 2021 [36]	China, Asia	Case-control	CIMT ≥0.9 mm and ≤1.2 mm	Serum	qRT-PCR	47.19	110 (51)	68 (33)	53.43 ± 4.59	52.69 ± 3.74	miR-18a-5p	Upregulated	Low	Low
Wang et al., 2020 [24]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	43.98	102 (48)	89 (36)	58.96 ± 9.04	57.35 ± 7.48	miR-374	Upregulated	Moderate	Low
Wang et al., 2021 [25]	China, Asia	Case-control	CIMT >0.7 mm	Serum	qRT-PCR	48.33	110 (52)	70 (35)	53.33 ± 5.70	54.61 ± 8.60	miR-675-3p	Upregulated	Low	Low
Xu et al., 2021 [26]	China, Asia	Case-control	CIMT >0.9 and <1.2 mm	Serum	qRT-PCR	52.17	86 (49)	75 (35)	59.70 ± 5.74	58.72 ± 5.03	miR-637	Downregulated	High	Moderate
Zhang et al., 2021 [27]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	52	90 (49)	85 (42)	64.46 ± 7.30	63.71 ± 7.15	miR-211-5p	Downregulated	Moderate	Low
Zhao et al., 2021 [28]	China, Asia	Case-control	CIMT ≥0.9 mm and <1.2 mm	Serum	qRT-PCR	44.64	88 (34)	80 (41)	62.80 ± 7.53	63.18 ± 7.57	miR-192-5p	Upregulated	Moderate	Low

CIMT, carotid intima-media thickness; **F**, female; **IMT**, intima-media thickness; **IQR**, interquartile range; **MiRNA**, microRNA; **N/A**, not available; **qRT-PCR**, quantitative real-time polymerase chain reaction; **QUADAS-2**, Quality Assessment of Diagnostic Accuracy Studies 2; **SD**, standard deviation.

[a] Data are presented in mean ± SD or median (IQR).

control subjects) were included in this analysis (Fig. 2). The result showed a pooled Fisher's *Z*-transformation value of 0.87 (95% CI = 0.80–0.93). After back-transformation, the finding indicated that circulating miRNAs were strongly correlated with CIMT with a pooled coefficient of 0.701 (95% CI = 0.664–0.731). The heterogeneity level was moderate (I^2 = 58%). The funnel plot showed a rather asymmetrical distribution of studies (Fig. S2). Nevertheless, the Egger's test result revealed no potential publication bias (Z = 0.47; p = 0.642).

3.4.2. Sensitivity analysis

The leave-one-out sensitivity analysis revealed that the pooled correlation coefficient was robust and not affected by any single study. Based on the forest plot (Fig. 2), one study by Xu et al. [26] was identified as an outlier. The result of sensitivity analyses by removing the outlier and studies with a moderate and high risk of bias showed no substantial change in the pooled coefficient (Table S6).

3.5. Accuracy of circulating miRNAs in detecting subclinical carotid atherosclerosis

3.5.1. Meta-analysis and publication bias

This diagnostic accuracy meta-analysis included thirteen studies with a total of 2272 samples (1270 patients and 1002 control subjects). Two studies by Guo et al. [22] and Huang et al. (2016) [23] were not included since sensitivity and specificity values were not reported. The results (Figs. 3 and 4) showed that circulating miRNAs could detect subclinical carotid atherosclerosis with a pooled sensitivity of 85% (95% CI = 80%–89%), specificity of 84% (95% CI = 78%–88%), PLR of 5.19 (95% CI = 3.97–6.80), NLR of 0.18 (95% CI = 0.13–0.23), DOR of 29.48 (95% CI = 21.15–41.11), and an AUSROC curve of 0.91 (95% CI = 0.88–0.93). This AUSROC value suggests that circulating miRNAs had an outstanding discriminatory power. The level of heterogeneity in all analyses was moderate to high (I^2 > 50%). The included studies presented a varied range of cut-off values of circulating miRNAs, which may potentially cause a threshold effect. Nonetheless, the Spearman's analysis showed an insignificant correlation coefficient (ρ = 0.46; p = 0.110), suggesting that the heterogeneity was unlikely to be caused by a threshold effect. The Deeks' funnel plot analysis indicated no potential publication bias (p = 0.20; Fig. S3).

3.5.2. Sensitivity analysis

The sensitivity analysis using the leave-one-out method demonstrated that no study had a substantial effect on the pooled sensitivity, specificity, and AUSROC curve. The bivariate boxplot (Fig. S4) indicated two study outliers by Huang et al. (2018) [30] and Li et al. [31] The result of sensitivity analyses by excluding outliers and studies with moderate and high bias risk revealed no substantial change in the pooled diagnostic accuracies (Table S7). It is worth mentioning that there was also no overestimation of the sensitivity, specificity, and AUSROC curve of circulating miRNAs due to the moderate and high risk of bias studies.

3.5.3. Subgroup and meta-regression analyses

Results of the subgroup and meta-regression analyses are presented in Table 2. We found significant differences in the sensitivity and specificity of circulating miRNAs between studies that used a case-control and cross-sectional design (p = 0.02) and studies that used a serum and plasma specimen (p = 0.02). Meta-regression analyses on the year of publication (p = 0.04) and mean population age (p = 0.01) showed significant results, suggesting that these factors influenced the pooled sensitivity and specificity of circulating miRNAs. The regulation mode of miRNAs, % female, and study sample size did not significantly affect the pooled diagnostic accuracies.

3.6. Network visualisation and enrichment bioinformatics analysis

The target gene network of the identified miRNAs is visualised in Fig. 5. The 15 included miRNAs were associated with several genes, with *SPARC*, *CCND1*, *WWTR1*, *VEGFA*, and *KCTD15* being the most targeted genes. *SPARC* and *CCND1* served as the target genes for seven miRNAs, while *WWTR1*, *VEGFA*, and *KCDT15* served as targets for eight miRNAs. The enrichment analysis in exploring diseases and pathways related to atherosclerosis is provided in Table S8. Fourteen out of the 15 miRNAs were significantly (p < 0.001) associated with vascular disease, suggesting the specific pathogenesis regulation of vascular diseases involving the identified miRNAs. Additionally, the miRNAs were found to be involved in atherosclerosis (p < 0.01), carotid artery disease (p < 0.01), and multiple pathways, including adipocytokine signalling (p < 0.001), the positive regulation of vascular endothelial cell proliferation, migration, adhesion, and growth factor receptor signalling (p < 0.01), as well as the negative regulation of endothelial cell apoptotic process (p <
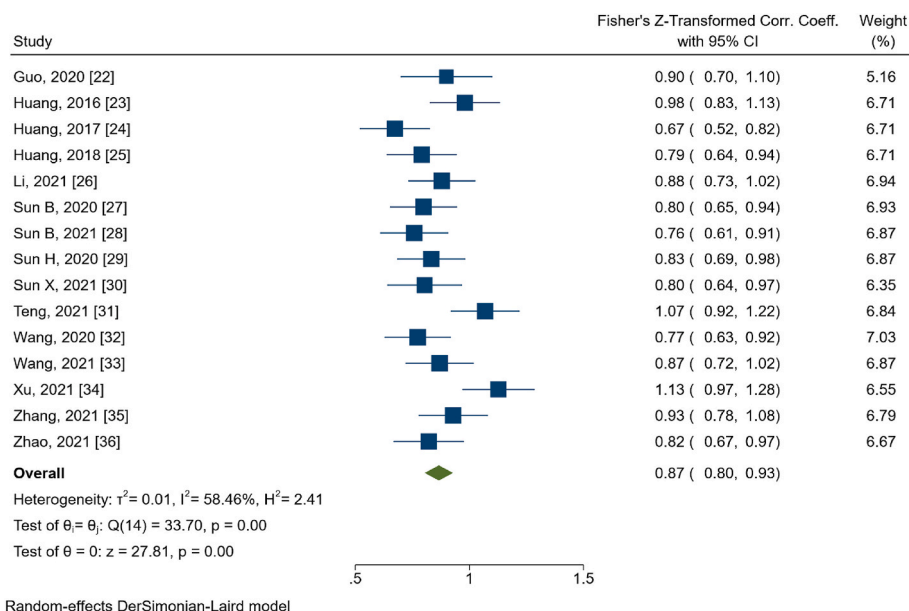

Study		Fisher's Z-Transformed Corr. Coeff. with 95% CI	Weight (%)
Guo, 2020 [22]		0.90 (0.70, 1.10)	5.16
Huang, 2016 [23]		0.98 (0.83, 1.13)	6.71
Huang, 2017 [24]		0.67 (0.52, 0.82)	6.71
Huang, 2018 [25]		0.79 (0.64, 0.94)	6.71
Li, 2021 [26]		0.88 (0.73, 1.02)	6.94
Sun B, 2020 [27]		0.80 (0.65, 0.94)	6.93
Sun B, 2021 [28]		0.76 (0.61, 0.91)	6.87
Sun H, 2020 [29]		0.83 (0.69, 0.98)	6.87
Sun X, 2021 [30]		0.80 (0.64, 0.97)	6.35
Teng, 2021 [31]		1.07 (0.92, 1.22)	6.84
Wang, 2020 [32]		0.77 (0.63, 0.92)	7.03
Wang, 2021 [33]		0.87 (0.72, 1.02)	6.87
Xu, 2021 [34]		1.13 (0.97, 1.28)	6.55
Zhang, 2021 [35]		0.93 (0.78, 1.08)	6.79
Zhao, 2021 [36]		0.82 (0.67, 0.97)	6.67
Overall		0.87 (0.80, 0.93)	

Heterogeneity: τ^2 = 0.01, I^2 = 58.46%, H^2 = 2.41
Test of $\theta_i = \theta_j$: Q(14) = 33.70, p = 0.00
Test of θ = 0: z = 27.81, p = 0.00

Random-effects DerSimonian-Laird model

Fig. 2. Forest plot of meta-analysis of correlation coefficient between circulating miRNAs and CIMT. **CI**, confidence interval; **CIMT**, carotid intima-media thickness; **Corr. Coeff.**, correlation coefficient; **MiRNAs**, microRNAs.
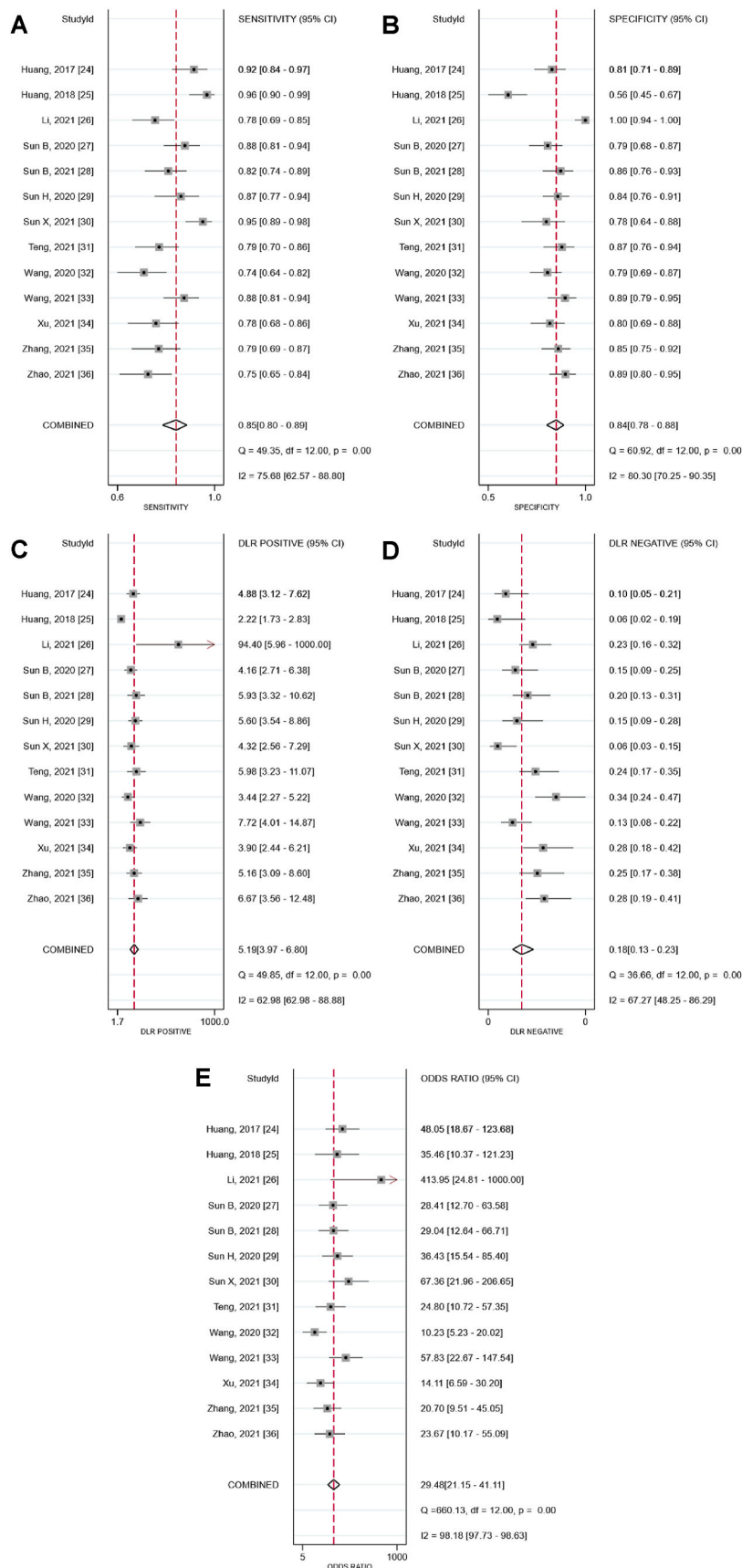
Fig. 3. Forest plots of diagnostic accuracy meta-analysis of circulating miRNAs in detecting subclinical carotid atherosclerosis. (**A**) Sensitivity, (**B**) Specificity, (**C**) PLR, (**D**) NLR, (**E**) DOR. **CI**, confidence interval; **DLR**, diagnostic likelihood ratio; **DOR**, diagnostic odds ratio; **MiRNAs**, microRNAs; **NLR**, negative likelihood ratio; **PLR**, positive likelihood ratio.
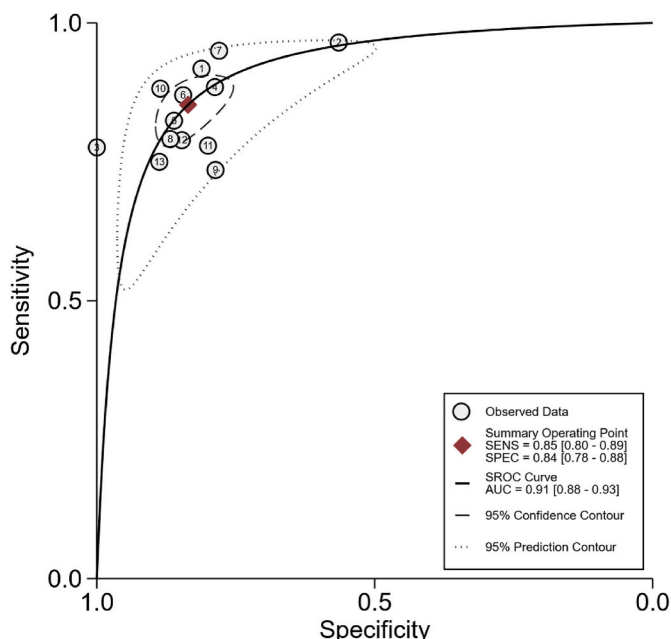
Fig. 4. AUSROC curve of circulating miRNAs in detecting subclinical carotid atherosclerosis. **AUC**, area under the curve; **AUSROC**; area under the summary receiver operating characteristic; **MiRNAs**, microRNAs; **SENS**, sensitivity; **SPEC**, specificity; **SROC**, summary receiver operating characteristic.

Table 2
Subgroup and meta-regression analyses for diagnostic accuracy meta-analysis of circulating miRNAs in detecting subclinical carotid atherosclerosis.

Subgroup and Meta-Regression Covariates	Number of Studies	Total Sample Size	% Pooled Sn (95% CI)	% Pooled Sp (95% CI)	p-value
Study Design					
Case-control	11	1932	83 (79–87)	85 (81–89)	ref.
Cross-sectional	2	340	95 (90–99)	70 (56–84)	0.02
Specimen					
Serum	11	1932	83 (79–87)	85 (81–89)	ref.
Plasma	2	340	95 (90–99)	70 (56–84)	0.02
Regulation Mode					
Upregulated	9	1606	85 (80–90)	84 (78–90)	ref.
Downregulated	4	666	86 (78–94)	82 (73–91)	0.93
Meta-Regressions					
Year of publication	13	2272	85 (80–89)	84 (78–88)	0.04
% female	13	2272	86 (80–89)	85 (78–88)	0.58
Sample size	13	2272	87 (80–89)	86 (78–88)	0.25
Mean population age	13	2272	88 (80–89)	87 (78–88)	0.01

CI, confidence interval; **MiRNAs**, microRNAs; **ref.**, reference value; **Sn**, sensitivity; **Sp**, specificity.
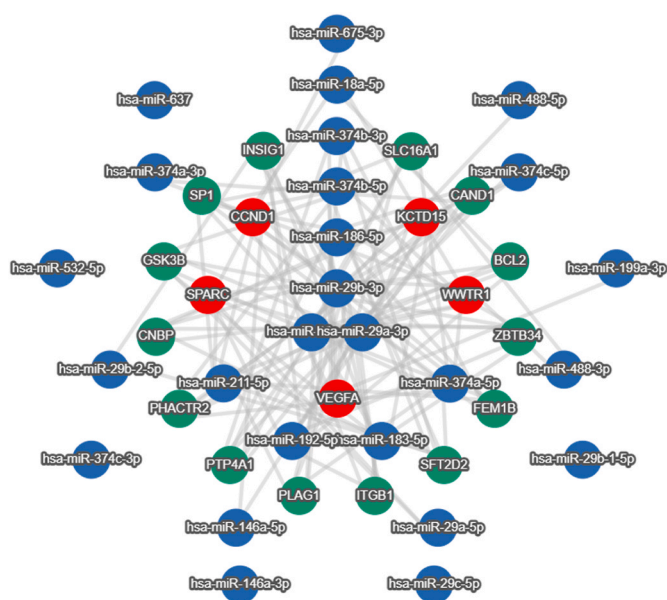

Fig. 5. Network visualisation of the interaction between the 15 identified circulating miRNAs and their target genes. Blue circle indicates the type of miRNA. Red and green circles indicate the target genes. Red circle indicates the most targeted genes. Grey line indicates an interaction between the miRNA and the target gene. **hsa**, *Homo sapiens*; **MiRNAs**, microRNAs.

0.001). MiR-29a, miR-29b, miR-29c, and miR-374 were involved in all of the identified pathways.

4. Discussion

4.1. Main findings

Our meta-analysis showed that miRNAs have excellent diagnostic performance for subclinical carotid atherosclerosis with a pooled sensitivity of 85% and specificity of 84%. This finding can be explained by the strong correlation between circulating miRNA expression and CIMT. CIMT is a biomarker for subclinical atherosclerosis, and patients with elevated CIMT are at high risk of future cardiovascular events [4]. MiRNAs may become inflammatory markers that play an essential role in the development of subclinical atherosclerosis [8]. Our findings are linear with previous meta-analyses using miRNAs for diagnosing ischemic stroke [37] and coronary artery disease [38].

We found a significant difference in the diagnostic accuracy between serum and plasma miRNAs. This finding is because the RNA sequence length of plasma contains mostly miRNA-related sequences, with plasma containing more proteins than serum [7,39]. More miRNAs are also detected in plasma than in serum, which may be attributed to platelet contamination, red blood cells, white blood cells, hemolysis, and qPCR inhibitors [40].

Our meta-regression showed that % females and sample size did not have a significant influence on the effect sizes. This result showed that miRNAs could be applied for detecting subclinical carotid atherosclerosis in both sexes. These results are supported by another study that found miRNAs are expressed at a certain level to maintain their expression level throughout the life span in both sexes [41]. We also found that age was associated with the diagnostic accuracy of circulating miRNAs. This finding might be due to the fact that miRNAs are significantly decreased in abundance in older individuals compared to young individuals [42]. This can happen due to biomechanical and structural alterations of the vascular system in older people.

4.2. Molecular mechanism of miRNA expression on atherosclerosis

Biogenesis of human miRNA involves consecutive cleavage events in the cell nucleus and subsequently in the cytoplasm. These processes are performed by two ribonuclease III endonucleases, called Drosha and Dicer. The miRNA gene is then transcribed into primary miRNA. Afterwards, primary miRNA is modified and undergoes several steps of maturation, from precursor miRNA, to miRNA duplex, and ultimately forms mature miRNA. Functional miRNAs will act on their target recognition (i.e., messenger RNA [mRNA]), which is known to be highly specific depending on its complementary base-pairing with the seed region (residues 2–8 at the 5' end) of the miRNA [43,44].

MiRNAs have the potential to regulate atherosclerosis biomolecular mechanisms. Some perform stabilising characteristics, while others perform destabilising characteristics [45]. We added bioinformatics analysis to explain the complexities encountered in the association of miRNAs in each stage of atherosclerosis formation and related events, which found that the identified miRNAs have a significant involvement in regulating the molecular pathways of carotid atherosclerosis pathogenesis, showing their potential role as biomarkers of the disease. The upregulation and downregulation of miRNA expression indicate a relationship between pathways and molecular interactions of certain genes during the development of atherosclerosis. We found that miRNAs play a role in the positive regulation of blood vessel endothelial activation and the negative regulation of its apoptosis. Even if these mechanisms were still not fully understood, the imbalance of endothelial activation could lead to the impairment of endothelial function [46]. The increase of vascular endothelial growth factor and adhesion of cell-substrate leads to increased CIMT, which further transforms into atherosclerosis [47].

MiRNAs may induce transcriptional repression or target mRNA degradation, with approximately >60% of all protein-coding genes estimated to be directly regulated by miRNAs [48]. Our bioinformatics analysis revealed the identified miRNAs had shared gene targets, mainly *SPARC*, *CCND1*, *WWTR1*, *VEGFA*, and *KCTD15*, that code for unique proteins. Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40, is a matrix-associated protein that elicits changes in cell shape, inhibits cell-cycle progression, and influences the synthesis of extracellular matrix [49]. Studies have found that SPARC is upregulated in high low-density lipoprotein cholesterol (LDL-C) and downregulated in low LDL-C levels. The secretion of SPARC into circulation may induce oxidised LDL, which triggers inflammation and vascular smooth muscle cell (VSMC) proliferation to initiate atherosclerosis [50]. Cyclin D1 (CCND1) protein is the regulatory subunit of a holoenzyme which could activate VSMC proliferation, migration, and expression. CCND1 was also associated with the upregulation of pro-inflammatory markers, such as IL-1β, IL-6 and IL-8 [51]. WW domain containing transcription regulator 1 (WWTR1) are proteins regulated by various stimuli and mediated by several pathways to initiate atherosclerosis development [52]. Vascular endothelial growth factor A (VEGFA) protein is a member of the platelet-derived/vascular endothelial growth factor family. VEGFA induces the proliferation and migration of vascular endothelial cells and also decreases the activity of plasma lipoprotein lipase, resulting in the accumulation of triglycerides in large lipoprotein granules, leading to atherosclerosis promotion [53]. Potassium channel tetramerisation domain containing 15 (KCTD15) protein plays an important regulatory role in energy balance, with its expression found to be upregulated when cells overexpressed obesity-related genes [54]. Knowing these target genes and their corresponding coded proteins may also expand the opportunity for developing novel drugs used in the early intervention of carotid atherosclerosis.

4.3. Applicability of circulating miRNAs as diagnostic tools for subclinical carotid atherosclerosis

Despite advances in lifestyle management and drug therapy,

atherosclerosis remains the major cause of high morbidity and mortality rates from cardiovascular diseases in industrialised countries [55]. Hence, there is a great need for reliable diagnostic biomarkers and effective treatment alternatives to reduce its burden, especially early disease detection [7]. As previously stated, miRNAs are involved in cell-specific physiological functions and the molecular mechanisms of pathologies in humans, including atherosclerosis. Thus, circulating miRNAs are promising biomarkers for the early detection of atherosclerosis, particularly miR-675-3p, which showed the highest diagnostic performance [7,25].

It was established that intracellular miRNAs are released into body fluids, including blood circulation [56]. Circulating miRNAs remain stable in serum and other body fluids as they are protected from degradation. One study argued that miRNA stability is induced by lipoprotein complexes [57]. Stability is considered one important aspect for a biomarker clinical development [58]; hence, circulating miRNAs, supported with their high sensitivity and specificity, are suitable as novel candidate biomarkers, also supported with their. In the past decade, a large amount of data was presented demonstrating that dysregulated levels of circulating miRNAs are strongly associated with the presence or absence of atherosclerosis or cardiovascular disease [59]. However, the detection of mechanisms triggering the dysregulation and the putative effects of the shifts in circulating miRNA levels continues to present challenges.

The level of miRNA expression can be measured by various methods, including microarray analysis, qRT-PCR, Northern blots, in situ hybridisation, solution hybridisation, and so on. Still, qRT-PCR is the most widely used technique with acceptable sensitivity and specificity [60]. Another technique, i.e., probe-based qPCR, offers the benefit of multiplexing, in which probes with several fluorophores are used inside the same reaction to detect various target sequences. This method significantly reduces the number of samples needed and the price of the reagents required for each reaction [61]. Together with the recent advances of miRNA measurement techniques, the applicability of miRNAs in large-scale screening may become more cost-effective since they allow researchers to recognise the gene expression quickly [62]. It can also be a preventive strategy for reducing health expenses in the future, knowing that carotid atherosclerosis causes adverse complications, such as stroke. Moreover, considering the overall healthcare, the annual cost to society of atherosclerotic disease has reached more than US$ 2000 [63]. In addition, blood tests to collect serum and plasma are considered minimally invasive and have been used in daily practice, making circulating miRNAs feasible biomarkers to be implemented. Considering the mean age distribution of subclinical carotid atherosclerosis patients from our included studies, it can be argued that the preferred time for the early disease detection using miRNAs is at the age of 50 years or older. The test may also be continued regularly since carotid atherosclerosis progresses with time [64].

4.4. Strengths and limitations

To our knowledge, this is the first systematic review and meta-analysis that thoroughly explored the role of circulating miRNAs as biomarkers of subclinical carotid atherosclerosis. We performed sensitivity analyses and found no confounding variables that could affect the pooled results. Subgroup and meta-regression analyses were also conducted to search for potential variables that caused heterogeneity in the analyses. Furthermore, our study incorporated bioinformatics analysis, further strengthening the role of circulating miRNAs in the development of carotid atherosclerosis.

Although we have made every effort to provide the best possible quality of the study, we acknowledged that there are still some limitations. First, our meta-analysis presented a moderate to high heterogeneity between studies. However, we identified that this heterogeneity most likely arose due to the differences in the study design, type of miRNA specimen, year of publication, and age of patients between

studies. In addition, there were no significant changes in the pooled results after performing sensitivity analyses, indicating that the results were stable. Second, the identified miRNAs were still varied, and thus conducting subgroup analyses were not possible, which we are certain that this is also the cause of the high heterogeneity in our analyses. Finally, the generalizability of this study's findings might be limited as all included studies were conducted in China.

5. Conclusion and recommendations

This meta-analysis supports the use of circulating miRNAs as novel tools in subclinical carotid atherosclerosis detection, given their excellent diagnostic accuracy. By performing bioinformatics analysis, we provided a better understanding of the role of miRNAs in the pathogenesis of atherosclerosis through multiple pathways along with their key target genes, such as *CCND1*, *KCTD15*, *SPARC*, *WWTR1*, and *VEGFA*, which may serve as a theoretical basis for further drug development for early intervention. Nevertheless, considering that all included studies were conducted in China, the current evidence should be carefully interpreted, particularly when it is implemented in other regions. We suggest future studies with large-scale and more heterogeneous populations to validate our findings and focus on comparing the diagnostic ability of circulating miRNAs for subclinical carotid atherosclerosis among different races, ethnicities, age groups, study designs, and other potential confounding factors that could not be identified in this study, such as patient's comorbidities, mainly metabolic (e.g., diabetes mellitus, obesity, and hypertension) and other atherosclerotic diseases (e.g., coronary artery disease and peripheral artery disease). Concurrently, researchers are encouraged to conduct sophisticated studies on clarifying which specific circulating miRNAs can accurately diagnose subclinical carotid atherosclerosis and determining their optimal threshold value that is most suitable for daily practice. Further studies may also design a specific panel consisting of more than one type of miRNA to improve the diagnostic performance of miRNAs for subclinical carotid atherosclerosis compared to the use of a single miRNA.

Sources of funding

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Authors' contributions

BSW and VV conceptualized the idea for the project and designed the methodology. BSW, VV, FMA, MSSA, and MVA administered the study protocol. BSW, VV, FMA, MSSA, MVA, and APW performed the literature search and study screening, data acquisition, and risk of bias assessment. BSW undertook the formal statistical analysis and visualised and interpreted the findings. VV conducted the bioinformatics analysis. CDKW, HS, MYA, and MSR performed extensive research on the topic and provided critical revisions to the manuscript. BSW, VV, FMA, MSSA, MVA, and APW drafted the initial manuscript. BSW, CDKW, HS, MYA, and MSR reviewed and validated the final manuscript, and BSW edited the manuscript for final submission. The whole project was supervised by CDKW and HS. All authors have read and approved the final manuscript for publication.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2023.102860.

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