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Review

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Cell-free DNA as a potential biomarker in stroke: a comprehensive review of observational studies

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Abstract

Stroke is an abrupt loss of brain function, which is caused by the interruption of blood flow to the brain. Several blood biomarkers have been evaluated for the assessment of stroke severity and outcome. However, their roles remain limited in clinical practice. Circulating cell-free deoxyribonucleic acid (DNA) has emerged as a potential biomarker of stroke, as reported from several animal and human studies. In this study, we aim to review the prognostic values of cell-free DNA in stroke from all relevant cohort studies. The PubMed database was searched using keywords, "cell-free DNA" and "stroke" for relevant articles. Twelve studies (n = 946 patients) are included in the final analysis. While the prognostic values of cell-free DNA in predicting functional outcomes and hospital mortality after different types of stroke were highlighted in many studies, the inconsistency in methods hinders comparability between studies. Overall, the knowledge about the potential prognostic ability of cell-free DNA in stroke remains limited and conflicting. More robust studies with consistent methods are needed.

Keywords: Cell-free DNA, stroke, nucleosome, biomarker

INTRODUCTION

Stroke is associated with significant morbidity and mortality and is noted to have a growing incidence worldwide, due to the increasing prevalence of cardiovascular associated diseases[1]. Cerebral ischemia is a detrimental neurological event where temporary or permanent depletion in blood flow causes injury in the brain[2]. The consequent interaction of pathophysiological events such as inflammation, excitotoxicity, and apoptosis cause tissue damage that often results in a compromise of the blood-brain barrier[2] and release of various neurobiological markers into circulation[3-5]. To date, various biomarkers have been discovered to support the diagnosis of stroke, however, none have acquired sufficient sensitivity or specificity to find a place in clinical practice[6].

First reported in 1948 by Mandel and Metais, cell-free DNA (cfDNA) has become one of the centralized subjects for investigation in medical field research[7]. Cell-free DNA consists primarily of double-stranded nuclear DNA and mitochondrial DNA. Studies suggest that 90% of cfDNA is associated with exosomes[8], where they are chromatinized and packaged into large extracellular vesicles[9]. Cell-free DNA circulates freely in blood plasma and is highly fragmented[7]. Thus, cfDNA can be collected during routine blood tests[7]. Its ability in becoming non-invasive, low cost, and simple in diagnosing diseases renders cfDNA an idealistic biomarker[7]. Recent papers have suggested its usage within fields such as oncology, fetal prenatal and physical activity monitoring[7], with several papers hinting at its potential in diagnosing and predicting prognosis in patients with stroke[10,11]. Despite the increase in the number of studies suggesting cfDNA's potential as a clinical biomarker, its origin, mechanism of release during a stroke, clearance, and physiological role remain widely unknown. The understanding of cfDNA origin and release mechanism can be advanced through studying cfDNA fragment size, which ranges from 150 bp to sizes larger than 10 kbp[12,13]. Fragments of multiples of 150 bp originate from apoptotic processes, specifically the endogenous cleavage of chromatin DNA into internucleosomal fragments. Larger fragments (> 10 kbp) are believed to originate from necrotic processes[14].

Previous studies[15] have demonstrated approaches where analysis of blood cfDNA successfully differentiated ischemic stroke (IS) patients from stroke mimics. Successful candidates of diagnostic blood biomarkers of stroke should also be capable of efficiently differentiating between various stroke subtypes[5]. Although there have been various discoveries of biomarkers specific to subtypes of stroke, the role of cfDNA as a novel biomarker necessitates further exploration in its association with stroke subtypes. Such biomarkers could further be utilized diagnostically in non-invasively assessing stroke severity, which may vary between subtypes as well. Since the therapeutic window of stroke is narrow, occurring 3-6 h after onset of symptoms, the timely detection of severe patients and rapid implementation of the corresponding therapeutic measures have immense prognostic significance[16,17]. Furthermore, the clinical relevance of biomarkers could be crucial in predicting mortality or functional outcomes of patients, which necessitates further investigation of cfDNA in the context of stroke. Despite the elevation of blood cfDNA during various pathological events in the body[18], conventionally, cfDNA has not gained importance as an ideal marker with specificity to a disease like stroke. Hence, this paper aims to examine the prognostic value of cfDNA in stroke through an examination of existing papers. The secondary objective is to explore the potential diagnostic values and to appraise the consistency in methods for measuring cfDNA.

CRITERIA FOR CONSIDERING STUDIES FOR REVIEW

The PubMed database was searched using keywords, "cell-free DNA" and "stroke" from inception to December, 2019. Inclusion criteria were limited to cohort or observational study on humans concerning cfDNA as a biomarker in stroke. Articles were excluded if they were non-human studies, comprised general reviews or editorials, and did not investigate cfDNA in the context of stroke. The references of the included articles were screened for further relevant studies. Data regarding: patient demographics, biological fluid

collected, time of sampling, methods employed to measure DNA levels, clinical outcomes, and the values of cfDNA levels between case and control groups were extracted onto a spreadsheet (Excel v14, Microsoft).

CURRENT RESEARCH STATUS

Out of 165 articles initially retrieved, 6 articles were deemed to be relevant. The references of the included studies were further screened for relevant articles, and 6 more studies were additionally added. Overall, 12 studies (n = 946 patients), are included in the final analysis. Article characteristics are presented in Table 1. The prognostic/diagnostic values of blood cfDNA levels in stroke are presented in Table 2.

Evidence for use of cfDNA in stroke recognition

Several studies investigated the ability of cfDNA in recognizing stroke. Tsai *et al.*[19] showed a significant elevation of plasma nuclear DNA and mitochondrial DNA in patients with acute IS (n = 50) compared to those in the control group (n = 50) who had no clinical evidence of stroke within one year. Blood samples were collected within 48 hours of stroke onset[19]. O'Connell *et al.*[15] reported similar elevation of peripheral blood cfDNA levels upon admission in patients suffering an acute IS (n = 43) relative to those diagnosed as stroke mimics (n = 20). These observations suggest that cfDNA may be diagnostically useful for the identification of IS during the acute phase of care. It is noteworthy in a study by Bustamante *et al.*[11] that cfDNA levels upon admission trended higher in acute IS patients (n = 54) compared to healthy controls (n = 15), yet the result was not significant. Rainer *et al.*[20] analyzed plasma DNA for the β-globin gene in a prospective studythat recruited patients presenting with stroke-like syndromes. Plasma β-globin DNA was significantly higher in patients suffering a hemorrhagic stroke (n = 118) than those without (n = 79), suggesting its ability to discriminate hemorrhagic from a non-hemorrhagic stroke[20].

Evidence for use of cfDNA in assessing stroke severity

DNA is released into the serum as a cell death marker in a time and severity dependent manner after the onset of stroke. Therefore, the concentration of cfDNA can be clinically useful for assessing stroke severity. In a cohort of patients suffering acute IS, the levels of cfDNA in plasma collected within 72 h after onset in patients with National Institutes of Health Stroke Scale (NIHSS) score >14 were significantly higher than the mild group (Score: 0-1) or the moderate group (score: 2-14)[21]. Vajpeyee *et al.*[10] assessed stroke severity by the NIHSS score in patients with IS. Plasma cfDNA correlated well with the severity of stroke at admission[10,22]. O'Connell *et al.*[15] reported similar findings that plasma cfDNA levels were positively correlated with NIHSS and infarct volume in 43 patients experiencing an acute ischaemic stroke. In 88 patients with IS, intracerebral hemorrhage, or transient ischemic attacks, Rainer *et al.*[23] found that cfDNA in plasma taken within 3 hours of symptom onset correlated well with the hemorrhagic volume, as measured by CT scan. In another study by Geiger *et al.*[24], the levels of nucleosomal DNA on day 3 post-admission were also significantly correlated with infarct size in patients with cerebral IS[24]. These observations suggest that cfDNA levels can be useful for stratifying injury severity during triage.

Evidence for use of cfDNA in predicting functional outcome

Cell-free DNA has also been investigated for its ability to predict outcomes in patients suffering a stroke. In acute IS patients receiving tissue plasminogen activator, baseline cfDNA level cfDNA < 302.75 kilogenome-equivalents/L (kg-equiv/L) was an independent predictor of neurological improvements at 48 hours, assessed by the NIHSS scale[11]. Geiger *et al.*[24] assessed the functional outcome using the Barthel Index in 63 patients with cerebral IS. Nucleosome DNA was significantly higher in stroke patients with severe functional impairments than stroke patients with moderate to low functional impairments[24]. In two studies recruiting a total of 80 patients with IS, Vajpeyee *et al.*[22] employed the modified Rankin scale (mRS) to determine the clinical outcome. They suggest the good utility of plasma cfDNA concentration upon admission as a predictor of a 3-month outcome in acute IS patients (P = 0.001) and neurological outcome after therapeutic interventions in the form of a mechanical thrombectomy or IV thrombolysis[10,22]. Lam *et al.*[25]

Table 1. cfDNA quantification and parameters correlating with blood cfDNA level in stroke

Author	Patient demographics	Biological fluids	Time of sampling from symptom onset	Methods	Cases DNA level (median unless otherwise stated)	Control DNA level	Significant correlation
Bustamante *et al.*[11] 2016	54 ischemic stroke Mean age: 77.0	Serum	< 4.5 h	DNA was extracted using MagNA Pure and Nucleic Acid Isolation Kit I (Roche Diagnostics) and measured using quantitative polymerase chain reaction for the β-Globin gene	Stroke patient: 408.5 (179-700.5) kg-equiv/L Neurological improvement in 48 h: 286.5 (152-688) kg-equiv/L No improvement in 48 h: 526 (382.8-927.5) kg-equiv/L	153.5 (66.9-700.5) kg-equiv/L	-
Geiger *et al.*[24] 2006	63 ischemic stroke Median age: 67.9	Serum	1-7 day	Nucleosomes were quantified using the Cell Death Detection ELISA^{Plus} (Roche Diagnostics). Two monoclonal antibodies to DNA and histones were used	In patients with BI ≥ 50: Day 1: 125 AU Day 2: 288 AU Day 5: 523 AU Day 7: 338 AU In patients with BI < 50: Day 1: 145 AU Day 2: 524 AU Day 3: 869 AU Day 7: 591 AU	-	Nucleosome concentration showed significant correlations for day 3
Geiger *et al.*[35] 2007	63 ischemic stroke Median age: 67.9	Serum	1-7 day	Nucleosomes were quantified using the Cell Death Detection ELISA^{Plus} (Roche Diagnostics). Two monoclonal antibodies to DNA and histones were used	In patients with BI < 50 Day 3: 525 ng/mL	-	-
Lam *et al.*[25] 2006	44 with negative neuroimaging results Median age: 74	Plasma	At admission	DNA was extracted by QIAmp DNA Blood kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene	mRS grade 0-2: > 800 kg-equiv/L (cutoff value)	-	Median plasma DNA concentration was significantly higher in patients of post-stroke mRS grades 3-6 when compared with patients of post-stroke mRS grades 0-2
O'Connell *et al.*[15] 2017	43 ischaemic stroke Mean age: 72.5 20 stroke mimics Mean age: 58.0	Plasma	Within 4.5 h	DNA was extracted by QIAmp DNA micro kit (Qiagen) and measured by quantitative polymerase chain reaction for the nuclear human TERT gene. A non-human 605 bp DNA fragment was used as a spike in control	-	-	Circulating cfDNA levels had a significant positive correlation with infract volume and was positively associated with post-stroke neutrophil count in patients experiencing AIS
Rainer *et al.*[23] 2003	70 ischemic stroke 11 intracerebral hemorrhage 7 transient ischemic attack Median age: 74	Plasma	Within 3 h	DNA was extracted by QIAmp DNA Blood kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene	Died, *n* = 11: 6,205 kg-equiv/L Survived, *n* = 77: 1,334 kg-equiv/L	-	Plasma DNA concentration correlated with mortality, the Glasgow Coma Score, cerebral hemorrhage volume, post-stroke modified Rankin Score and quality of life loss

Rainer *et al.*[20] 2007	118 ischemic stroke 35 hemorrhage 44 no acute neuroimaging changes Median age: 74	Plasma	Within 10 h	DNA was extracted by QIAmp DNA Blood kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene	Hemorrhage, n = 35: 1,725 kg-equiv/L Ischemic, n = 118: 1,050 kg-equiv/L mRS Grades ≥ 2: 1,025 kg-equiv/L Died: 1,625 kg-equiv/L Survived: 1,050 kg-equiv/L	-	Plasma DNA can predict presence of stroke (hemorrhagic *vs*. non-hemorrhagic etiology on neuroimaging), 6-month mortality and 6-month post-stroke mRS
Tsai *et al.*[19] 2011	50 acute ischemic stoke Mean age: 67.0	Plasma	Within 48 h	DNA was extracted by QIAmp DNA Blood kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene and MT-ND2 gene (Roche Lightcycler)	At admission: Nuclear DNA: 5393.4 ± 454.5 kg-equiv/L (mean) Mitochondrial DNA: 3045.2 ± 384.0 kg-equiv/L (mean) Poor outcome group: Nuclear DNA: 5575.2 ± 818.1 kg-equiv/L (mean) Mitochondrial DNA: 3120.9 ± 970.0 kg-equiv/L (mean) Good outcome group: Nuclear DNA: 5120.7 ± 378.8 kg-equiv/L (mean) Mitochondrial DNA: 2333.1 ± 272.7 kg-equiv/L (mean)	At admission: Nuclear DNA: 3681.5 ± 197.0 kg-equiv/L (mean) Mitochondrial DNA: 1984.7 ± 166.7 kg-equiv/L (mean)	Plasma nuclear DNA and mitochondrial DNA are significantly higher in stroke patients than in the controls
Vajpeyee *et al.*[10] 2018	26 ischemic stroke Mean age: 62.88	Plasma	Within 6 h	DNA was extracted by QIAmp Circulating Nucleic Acid Kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene	-	-	Plasma DNA level correlated with severity of stroke at time of admission and poor outcome at 3 months
Vajpeyee *et al.*[22] 2020	54 ischemic stroke Mean age: 61.28	Plasma	Within 12 h	DNA was extracted by QIAmp Circulating Nucleic Acid Kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene	-	-	High cfDNA levels were associated with severity at time of admission and poor outcome
Valles *et al.*[21] 2017	243 ischemic stroke Mean age: 70.7	Plasma	Within 72 h	DNA concentration was measured in a fluorescence microplate reader after mixing plasma with the cell-impermeable DNA binding dye, SytoxGreen (Invitrogen)	cfDNA: 432.11 ± 9.95 ng/mL (mean)	cfDNA: 324.2 ± 13.44 ng/mL (mean)	High cfDNA levels were correlated with higher NIHSS score at onset and stroke severity at discharge. The levels of cfDNA were higher in patients with cardioembolic stroke *vs*. atherosclerotic or lacunar stroke
Wang *et al.*[8] 2013	21 aneurysmal SAH Median age: 52	CSF and plasma	Within 24 h	DNA was extracted by QIAmp DNA Blood kit (Qiagen) and measured by real-time quantitative polymerase chain reaction for the β-Globin gene and ND2 genes	Plasma nuclear DNA: 56.1 (50.7-173.5) ng/mL Plasma mitochondrial DNA: 16.9 (5.4-39.6) ng/mL CSF nuclear DNA: 157.0 (65.7-272.5) ng/mL CSF mitochondrial DNA: 39.2 (19.6-74.1) ng/mL	Plasma nuclear DNA: 26.7 (17.6-42.7) ng/mL Plasma mitochondrial DNA: 9.8 (6.5-15.9) ng/mL CSF nuclear DNA: 1.2 (0.3-3.6) ng/mL CSF mitochondrial DNA: 4.2 (1.5-5.3) ng/mL	In patients with poor outcome, the CSF nuclear and mitochondrial DNA levels were significantly higher on Days 1 and 4, and plasma nuclear DNA levels were significantly higher from Day 8 to Day 14

kg-equiv/L: kilogenome-equivalents/L; BI: barthel index; AU: arbitrary unit; mRS: modified rankin scale; cfDNA: cell free DNA; AIS: acute ischemic stroke; SAH: subarachnoid hemorrhage; CSF: cerebrospinal fluid

Table 2. Prognostic/diagnostic values of blood cfDNA levels in stroke

Author	Outcome	Marker and cutoff value	Sensitivity, % (95%CI)	Specificity, % (95%CI)	Area under ROC (95%CI)	Odds ratio
Bustamante *et al.*[11] 2016	48 h neurological improvement	cfDNA: < 302.75 kg-equiv/L	81 (68.57-89.37)	55 (41.4-67.38)	-	43.8 (3.1-620.9) (adjusted OR)
Geiger *et al.*[35] 2007	Non-recovery, BI < 100	Plasma nucleosome cfDNA on day 3 after admission: 560 ng/mL	52.6 (40.94-65.12)	100 (94.31-100)	-	-
Lam *et al.*[25] 2006	6-month post-stroke mRS, Grades 0-2	Plasma ncfDNA: > 800 kg-equiv/L	42 (28.35-56.75)	100 (91.96-100)	0.742 (0.588-0.862)	-
Rainer *et al.*[23] 2003	Hospital mortality	Plasma ncfDNA: > 1,400 kg-equiv/L	100 (95.9-100)	74.4 (64.63-82.66)	0.89 (0.80-0.94)	Mortality: 1.6 (1.1-2.4; $P = 0.03$) 6-month post-Rankin Score > 2: 1.8 (1.0-3.3; $P = 0.05$)
Rainer *et al.*[20] 2007	Stroke type: hemorrhagic stroke *vs*. (ischemic stroke and patients without acute neuroimaging changes)	Plasma ncfDNA: > 2,500 kg-equiv/L	31 (25.06-37.93)	83 (77.29-87.74)	-	4.24 (1.88-9.56; $P = 0.0011$)
Vajpeyee *et al.*[10] 2018	Better outcome at 3 months for patients who underwent IVT and/or mechanical thrombectomy	cfDNA: < 10,000 kg-equiv/L	78	83	0.79 (0.67-0.92; $P = 0.02$)	40.33 (1.50-293.25; $P = 0.000$)
Wang *et al.*[8] 2013	6-month post-stroke mRS, score ≥ 3	CSF ncfDNA: 85.1 ng/mL	89	75	-	-
		CSF mcfDNA: 31.4 ng/mL	89	100		

cfDNA: cell free DNA; kg-equiv/L: kilogenome-equivalents/L; BI: barthel index; ncfDNA: nuclear cell free DNA; IVT: intravenous thrombolysis; mRS: modified rankin scale; CSF: cerebrospinal fluid; mcfDNA: mitochondrial cell free DNA

also evaluated the 6-month outcome of their patients with the mRS scale. In patients with negative neuroimaging results, there was a significant difference in plasma DNA levels at admission between the good outcome (mRS 0-2) group and the poor outcome (mRS 3-6) group[25]. On the other hand, Wang *et al.*[26] found that in patients with acute spontaneous aneurysmal subarachnoid hemorrhage stroke, higher levels of cfDNA in plasma and nuclear and mitochondrial DNA in CSF upon admission are associated with the worse 6-month clinical outcome on the mRS scale. Overall, these findings suggested cfDNA as a good prognostic marker for stroke.

Evidence for use of cfDNA in predicting mortality

One of the main patient-important outcomes which have been examined in existing studies is mortality. In one of the preliminary studies describing the relationship between cfDNA and patient-important outcomes, Rainer *et al.*[23] described a 100% sensitivity and 74.4% specificity in using cfDNA as a prognostic biomarker for both ischaemic and hemorrhagic stroke[5]. Under categorical analysis, a cfDNA level of > 1,400 kg-equiv/L indicated a significant 60% increase of odds risk for the event of mortality at 6-months. Furthermore, in an updated study by the same group in 2007, a significant difference between cfDNA at 48 h post-stroke was as strong a predictor of 6-month mortality, with a 50% lower cfDNA levels for those without an event[20].

CURRENT APPLICATION OF CELL-FREE DNA IN STROKE

In this review, we summarize evidence that pertains to the biomarker prognostic value of cfDNA and stroke outcomes. Patient-important outcomes have been explored such as neurological outcomes (as assessed by mRS), infarction volume, and stroke severity at discharge. All of these factors were strongly

statistically significant in all the reported studies and demonstrated a high level of correlation. With a total of 484 patients from 7 studies[10,11,20,22,23,25,26], the evidence is quite compelling for cfDNA's predictive value of at-discharge and 3-month post-event mRS scale. With this in mind, it would be of benefit to consider the cfDNA as a simple test to adequately administer a more intensive post-discharge monitoring to stroke patients with poorer prognosis. Therefore, cell-free DNA has been proposed as another objective predictor of outcomes post-stroke. It can be analogously compared to troponin levels in myocardial infarction. The mechanism by which cell-free DNA increases in the plasma of patients suffering a stroke is still being studied. The stroke resulted from a complex cascade of events including cerebral ischemia, altered cerebral blood flow, inflammation, the production of reactive oxygen radicals, neuronal necrosis, and apoptosis[27-29]. Free DNA can be released from apoptotic cells, as observed in cancer[30,31] and trauma[32]. On the other hand, there is a possibility that other non-cerebral tissue pathologies associated with cell death may contribute to the increased cfDNA content in the plasma after stroke.

It is important to note that the majority of the studies report on ischaemic stroke. Out of the 12 articles, 10 articles (n = 781) reported on ischaemic stroke, whereas only 2 articles (n = 46) reported on hemorrhagic strokes - with one of the two studies being a mixed population with IS. Amongst the evidence for ischaemic strokes, 8 found a significant correlation between cfDNA and outcomes, such as those mentioned above. For the two articles examining hemorrhagic stroke, the correlations with cfDNA were significant; however, only one study performed a sensitivity analysis on their results and demonstrated that differences in cfDNA due to differing aetiologies. This suggests that although there is significant evidence for the correlation of cfDNA with ischaemic stroke, there remains a lack of data for both the correlation between hemorrhagic stroke and cfDNA. Furthermore, the prognostic potential of mitochondrial cfDNA was evaluated in only two studies, recruiting patients suffering from acute IS and subarachnoid hemorrhage[19,26]. Mitochondrial cfDNA is associated with the systemic and local immune responses, which have important roles in causing stroke as well as the progression of ischemic lesions[33,34]. However, the lack of data currently does not allow us to conclude mitochondrial cfDNA as a useful biomarker in stroke.

The benefit and clinical usefulness of cfDNA in predicting functional outcomes and long-term survival are apparent. The strength of using plasma cfDNA as a prognostic marker is that it is non-invasive and simple. It has also been demonstrated to be able to discriminate hemorrhagic from non-hemorrhagic stroke as well as being an independent predictor of the result of stroke in patients with negative neuroimaging[20,25]. Cell-free DNA can assist clinicians in patient examination and complement imaging tools to enhance the accuracy of stroke diagnosis. In addition, it can augment the diagnostic workup and help triage patients for intervention. In cases where imaging is negative or not indicated, cfDNA prognostic utility can assist the patients and clinicians in making informed decisions regarding invasive or medical treatment.

Despite the proven ability of cfDNA in predicting the prognosis of patients within the discharge and post-discharge setting, there remains conflicting evidence on its predictive value within the discharge setting. The notion remains true as studies conducted by Bustamante *et al.*[11] and O'connell *et al.*[15] indicated a weak correlation between cfDNA and NIHSS score. In contrast, these findings were not replicated in studies presented by Vajpeyee *et al.*[10] 2018, Vajpeyee *et al.*[22] 2020, and Valles *et al.*[21] 2017 as they have indicated a strong correlation between cfDNA and NIHSS. Thus, due to conflicting evidence presented in current studies, it remains unclear the predictive value of cfDNA and its correlation to NIHSS onstroke severity. However, differences in findings may be attributable to differences in baseline patient demographics used by these studies. Additionally, the lack of separation of ischaemic stroke and hemorrhagic stroke, regarding its correlation to NIHSS during an assessment may also contribute to the heterogeneity in findings. Other factors that may contribute to the conflicting outcomes also include the lack of analyses of factors such as sex and infarct volume. These factors are significant predictors of prognosis during post-event. Notably, differences in findings may be corrected if these factors are addressed in future studies, potentially leading to the production of stronger and more robust findings.

Methodological Considerations for Quantifying cfDNA

In some studies[11,24,35], cfDNA was measured in serum, while in others[10,15,19-23,25,26] it was measured in the blood plasma. It has been known that cfDNA is more abundant in serum than in plasma samples and there is a large variation of cfDNA in serum between patients. A major fraction of cfDNA in the blood serum might be formed during the coagulation cascade, which leads to the lysis of white blood cells[36,37]. Therefore, this effect can introduce errors to the obtained results. Geiger *et al.*[35] measured cfDNA in the serum of patients suffering an IS and found no statistically significant differences in the cfDNA levels between the severe and mild cases in the first day of the stroke, which was in contrast to the findings of other authors[24]. In addition, Wang *et al.*[26] measured the content of cfDNA in the cerebrospinal fluid (CSF) of patients suffering a subarachnoid hemorrhage. Interestingly, the cfDNA concentrations in the CSF, not in plasma, on admission were significantly increased in patients with worse outcomes. The observed differences in cfDNA level between plasma and CSF at the same timepoint can be explained by the different sources of cfDNA in these fluids and the differences in the dynamics of the blood-brain barrier and CSF-brain barrier integrity changes during a stroke[38,39]. Overall, the results between different sample types, such as a serum, plasma, or CSF, shouldn't be compared with one another[40].

Furthermore, inconsistencies in the sampling time severely limit the applicability and coalescing of evidence. The notion can predominantly be seen across all 12 studies, as there remains a lack of standardized sampling time protocol. The sampling time ranges significantly between zero (on admission) up to 72 h within the onset of symptoms. Sampling frequency also ranges from once to daily as long as the patient is admitted. Knowing that cfDNA is a transient molecule, it may be beneficial to standardize the collection time to ensure that cfDNA is captured within the time frame of rising, peak or falling levels. The timing of diagnosis is crucial since the therapeutic window for stroke is narrow after the onset of symptoms[16,17].

Another factor that limits the accuracy of biomarker interpretation is the use of different methods in extracting and measuring cfDNA. The majority of studies included in our analysis employed the QIAamp circulating nucleic acid kit for DNA extraction. The QIA kit has been reported to be highly efficient and produces high cfDNA yields[41-43]. While the ultimate goal was to quantify cell death, there were different protocols used across studies including the use of quantitative PCR, cell death detection enzyme-linked immunosorbent assay, and a nucleic acid immunofluorescent counterstain. The current cfDNA assay protocol in stroke is not standardized and there has been little investigation of consistency regarding the quantitative PCR methods used for cfDNA quantification. The widely-used technique to determine cfDNA concentration is quantitative PCR[10,11,19,20,22,23,25,26]. During the quantitative PCR process, the total DNA isolated from plasma or serum is eluted in a fixed volume of buffer. Then, qPCR targeting 1 or more loci, such as the β-globin gene for nuclear cfDNA and of the MT-ND2 gene for mitochondrial cfDNA, is performed using a fixed volume of eluent as input. The concentration of cfDNA in the sample is determined from the detection of these loci. This process is based on an assumption that DNA extraction efficiencies are similar between specimens. However, O'Connell *et al.*[44] demonstrated a large variation in extraction efficiencies between specimens, with a coefficient of variance of 28.9%. This effect can cause significant artificial variance with regards to downstream cfDNA quantification. To control for this potential confounder, an exogenous spike-in oligonucleotide fragment has been employed to normalize cfDNA levels[15]. Similar strategies using exogenous spike-in control to account for variance are often used in miRNA quantification, but the technique has yet to be adopted for quantification of cfDNA[45]. Other techniques include extracting DNA by nuclear monoclonal antibodies and the cfDNA content was measured using commercial ELISA kit[24,35]. A more effective approach using the integrity index has been introduced in studies investigating oncology patients[46,47].

The lack of standardization and appropriate controls hinders the use of cfDNA as a biomarker for stroke diagnosis and monitoring. Results between studies are not comparable due to differences in sample

processing methods, storage conditions, and techniques for both the extraction and quantification. This can lead to errors in determining cut-off points and assay sensitivity and specificity. Therefore, accurate and standardized quantification of cfDNA will aid the future clinical implementation of this approach. For instance, the use of an exogenous spike-in control is a step towards the right direction to limit artificial variance induced during DNA extraction. There are other potential confounders during blood collection, processing, and storage that causes DNA degradation and leukocyte enucleation. Different approaches to cfDNA extraction, storage, and assay have been described along with their benefits and disadvantages in detail by Wong *et al.*[48], El Messaoudi *et al.*[49], and Malentacchi *et al.*[50]. Efforts have been made to limit these variances, including the use of specifically designed blood preservatives for cfDNA analysis and the institutional specimen handling protocols for clinical blood collection[48,51]. It is perhaps beneficial in adhering to a standardized protocol to bolster the robustness of the current cfDNA in biomarker prognosis. Due to the inconsistency of biomarker collection and processing, conclusions and significance in results may be inherently limited.

CONCLUSION

The prognostic values of cell-free DNA in predicting functional outcomes and hospital mortality have been demonstrated in a limited number of studies. The inconsistency in the DNA extraction and quantification method hinders comparability between studies. This prompts future trials to conduct more robust cohort studies that may describe the most optimal collection times for stroke prediction as well as cfDNA processing that yields the most accurate prediction.

DECLARATIONS

Authors' contributions

Took part in the design of this paper: Tieu PT, Lee MH, Dhawan T, Nguyen HH, Afraz S, Chung J, Khan S, Yusuf I, Liu SSH
Performed the literature search, data extraction, and analysis: Tieu PT
Drafted the manuscript: Lee MH, Dhawan T, Nguyen HH, Afraz S, Chung J, Khan S
Provided material support and prepared the tables: Yusuf I, Liu SSH

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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