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A standardised methodology for the extraction and quantification of cell-free DNA in cerebrospinal fluid and application to evaluation of Alzheimer's disease and brain cancers

Petros Takousis [a,1], Alison S. Devonshire [b,*,1], Nicholas Redshaw [b], Louisa von Baumgarten [c,d], Alexandra S. Whale [b], Gerwyn M. Jones [b], Ana Fernandez-Gonzalez [b], Jan Martin [e], Carole A. Foy [b], Panagiotis Alexopoulos [f,g], Jim F. Huggett [b,h], Robert Perneczky [a,i,j,k,l]

[a] Ageing Epidemiology (AGE) Research Unit, School of Public Health, Imperial College London, London, UK
[b] Molecular and Cell Biology Team, National Measurement Laboratory, LGC, Teddington, Middlesex, UK
[c] Department of Neurology, University Hospital, LMU Munich, Munich, Germany
[d] Department of Neurosurgery, University Hospital, LMU Munich, Munich, Germany
[e] Department of Anaesthesiology and Intensive Care Medicine, Technical University Munich, Munich, Germany
[f] Department of Psychiatry, University of Patras, Rion Patras, Greece
[g] Department of Psychiatry and Psychotherapy, Technical University Munich, Munich, Germany
[h] School of Biosciences and Medicine, University of Surrey, Guildford, UK
[i] Department of Psychiatry and Psychotherapy, University Hospital, LMU Munich, Munich, Germany
[j] German Center for Neurodegenerative Diseases (DZNE) Munich, Munich, Germany
[k] Munich Cluster for Systems Neurology (SyNergy), Munich, Germany
[l] Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, UK

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ABSTRACT

Cerebrospinal fluid (CSF) is a source of diagnostic biomarkers for a range of neurological conditions. Cell-free DNA (cfDNA) is detected in CSF and differences in the concentration of cell-free mitochondrial DNA have been reported in studies of neurodegenerative disorders including Alzheimer's disease (AD). However, the influence of pre-analytical steps has not been investigated for cfDNA in CSF and there is no standardised approach for quantification of total cfDNA (copies of nuclear genome or mitochondria-derived gene targets). In this study, the suitability of four extraction methods was evaluated: QIAamp Circulating Nucleic Acid (Qiagen), Quick-cfDNA Serum & Plasma (Zymo), NucleoSnap® DNA Plasma (Macherey-Nagel) and Plasma/Serum Circulating DNA Purification Mini (Norgen) kits, for cfDNA extraction from CSF of controls and AD dementia patients, utilising a spike-in control for extraction efficiency and fragment size. One of the optimal extraction methods was applied to a comparison of cfDNA concentrations in CSF from control subjects, AD dementia and primary and secondary brain tumour patients. Extraction efficiency based on spike-in recovery was similar in all three groups whilst both endogenous mitochondrial and nucleus-derived cfDNA was significantly higher in CSF from cancer patients compared to control and AD groups, which typically contained < 100 genome copies/mL. This study shows that it is feasible to measure low concentration nuclear and mitochondrial gene targets in CSF and that normalisation of extraction yield can help control pre-analytical variability influencing biomarker measurements.

Abbreviations: AD, Alzheimer's disease; cfDNA, cell-free DNA; CNA, circulating nucleic acids kit; CSF, cerebrospinal fluid; dPCR, digital PCR; GE, genome equivalents; gDNA, genomic DNA; mt, mitochondrial; mtDNA, mitochondrial DNA; nc, nuclear; NS, NucleoSnap® DNA Plasma kit; PCP, Plasma/Serum Circulating DNA Purification mini kit; QSP, Quick-cfDNA Serum & Plasma kit.

* Corresponding author.
E-mail address: alison.devonshire@lgcgroup.com (A.S. Devonshire).

[1] Both authors contributed equally.

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Introduction

Extracellular nucleic acids in biological liquids, DNA in particular, and their role as putative biomarkers of disease have been studied extensively over the years (reviewed in [1–3] and elsewhere). To explain the high levels of circulating DNA in plasma and serum samples from disease cases in one of the earliest studies (in the 1960s), it was postulated that circulating DNA likely originates from tissue breakdown [4]. In a later study, serum DNA levels in half the number of cancer patient samples analysed were above the normal range determined therein [5] and further evidence of elevated circulating DNA levels in patients with different cancer types was subsequently reported [6,7]. Unequivocal proof of initial findings that DNA originating from cancer cells is detected in plasma [8] came in the mid-1990s, when oncogene mutations and microsatellite alterations associated with malignant cells were also found to be present in circulating DNA extracted from those patients [9–11]. Developments in the context of cancer research, paved the way for a rapid expansion in the field of non-invasive prenatal diagnostics [12].

Cell-free DNA (cfDNA) is a very sensitive marker for changes in cellular death rate and disruptions in homeostasis, since it can emanate from each dying cell in the body. Consequently, research on circulating nucleic acids has also found applications in the fields of organ transplantation, trauma and post-trauma monitoring, sepsis, myocardial infarction, stroke, diabetes mellitus, haematologic disorders and infectious agent detection [reviewed in [13]]. Indeed, the majority of analyses on extracellular nucleic acids have been conducted on blood components. However, a growing number of studies in the past two decades have addressed research questions involving the analysis of cfDNA in cerebrospinal fluid (CSF) [14–20]. CSF is the optimal body fluid to measure biomarkers of central nervous system (CNS) pathologies, such as neoplastic disease and neurodegenerative disorders, because it is produced in the brain and bathes the brain tissue. Reduced mitochondrial DNA (mtDNA) concentration in CSF is a putative biomarker of mild cognitive impairment and Alzheimer's disease (AD), proposed to be a biomarker for impaired mitochondrial biogenesis [18], which may be predictive of early stage disease before established CSF protein biomarkers amyloid-β and Tau are diagnostic [21].

Recently, significant progress has been made, through projects including Cancer-ID [22] and SPIDIA [23], in characterising the influence of pre-analytical steps such as blood sample collection and storage, nucleic acid extraction and quantification on cfDNA measurements [24]. Despite the potential advantages of measuring cfDNA in CSF to aid the diagnosis of CNS disorders, standardised isolation and quantification methods, and the influence of these factors on measurements of putative biomarkers such as mitochondrial cfDNA are yet to be defined. Drawing on previously developed approaches for characterising plasma cfDNA extraction and quantification [25], a two-phase approach (Fig. 1) was followed to evaluate cfDNA isolation and quantification in CSF and its impact on CNS biomarker validation. First, cfDNA extraction efficiency and potential bias associated with fragment size was evaluated for four commercially-available kits using an exogenous spike-in control material, "ADH plasmid" containing the *Arabidopsis* alcohol dehydrogenase gene sequence [25], and assays compared for nuclear and mitochondrial genes in pooled CSF samples of AD patients and control subjects. One of the best performing extraction methods was selected for the second phase, where cfDNA in individual CSF samples from patients with AD dementia, primary or metastatic brain tumours and healthy controls was quantified and levels of two mitochondrial genes and nuclear genomic DNA copies compared.

Materials and methods

Study participants

Patients with AD dementia ($n = 12$, mean age = 75.4 years) and healthy control subjects ($n = 10$, mean age = 57.8 years) with available CSF samples were identified in the biobank of the Department of Psychiatry and Psychotherapy of Technical University of Munich (TUM). AD patients met criteria of the National Institute of Neurological and Communicative Disorders and Stroke/AD and Related Disorders Association (NINCDS-ADRDA) [26]. Healthy controls were recruited from individuals undergoing elective surgery with spinal anaesthesia, had no history of neurological or psychiatric disorders, no subjective memory complaints, normal test results on the Mini-Mental-State Examination (MMSE) and normal results on the Beck Depression Inventory (BDI). CSF samples from patients with primary ($n = 8$; 2 × medullo-blastoma; 4 × astrocytoma; 2 × glioblastoma) and secondary ($n = 4$; 3 × Non-small-cell lung carcinoma; 1 × adeno-cancer of unknown origin) brain tumours were retrieved from the biobank of the Department of Neurology of Ludwig-Maximilians-Universität München (LMU). Participant characteristics are provided in Supplementary Table 1. The study was approved by the Ethics Committees of the medical faculties of TUM and LMU and was conducted according to the 1975 Declaration of Helsinki; all patients and controls gave written informed consent.

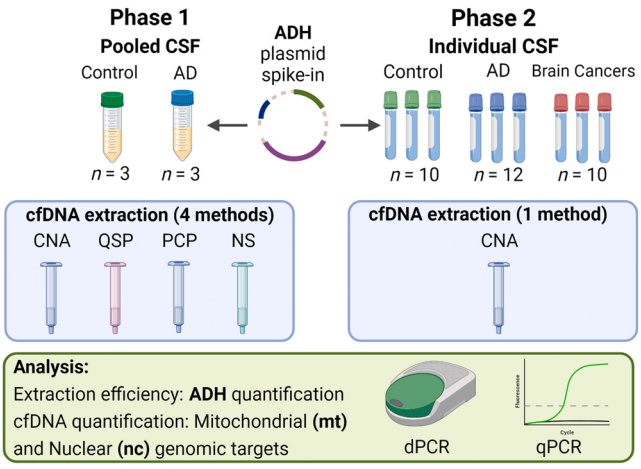

Fig. 1. Outline of study design for Phase 1 analysis using pooled CSF samples (A) and Phase 2 analysis using individual CSF patient samples (B).

CSF collection, protein assays and genotyping

In both the TUM and LMU cohorts, CSF (5–8 mL) was collected in sterile polypropylene tubes, using atraumatic cannulas placed in the L3/L4 or L4/L5 intervertebral space, and gently mixed. Serum and EDTA plasma samples for each subject were obtained by venous puncture. In the native CSF, determination of routine cytological and chemical parameters was performed. These parameters included leucocyte and erythrocyte cell counts, as well as glucose and lactate measurement, total protein content, CSF-serum ratios of albumin and immunoglobulin G, and screening for oligoclonal bands. Upon visual inspection, all CSF samples were clear and colourless. CSF cell-counting was performed (using both manual hemocytometer chamber and automated methods) and no sample showed abnormal results (i.e. leucocyte count > 5/mm^3) Total protein content was measured by turbidimetry after denaturation with trichloroacetic acid. The CSF was centrifuged for 15 min at 2000 × *g* and aliquots of the remaining CSF supernatants were initially stored at − 20 °C – within two hours from collection – and subsequently transferred to − 80 °C for long-term storage.

In AD dementia and control subjects, amyloid-β (Aβ)1–40 and 1–42, total-tau (T-TAU) and phosphorylated-tau181 (P-TAU181) levels in CSF were measured in duplicate using commercially available Enzyme-linked Immunosorbent Assays (ELISA) according to the manufacturers' instructions (Innogenetics, Ghent, Belgium) as described previously in greater detail [26,27]. Further details for AD cases and healthy control

participants are provided in Supplementary Table 1.

CSF sample preparation

In Phase 1, two sample pools were produced by mixing CSF material from healthy controls ($n = 10$, 'Pool C', total 20 mL) and AD patients ($n = 12$, 'Pool AD', total 18 mL). Fragmented ADH plasmid was added to each pool at 10^6 copies/mL CSF, then homogenised on a SpiraMixer at 4 °C for 30 min before aliquoting (12 × 1.1 mL aliquots for each pool). In Phase 2, individual CSF samples ($n = 10$ controls, $n = 12$ AD, $n = 12$ cancer) were also spiked with 10^6 copies/mL of fragmented ADH plasmid prior to extraction. All CSF samples were stored at − 80 °C.

Fragmentation of ADH plasmid

The pSP64 poly(A) plasmid (GenBank accession no. X65328.2) containing an *Arabidopsis thaliana* alcohol dehydrogenase gene (*ADH*) fragment (GenBank accession no. M12196) was digested as described previously [25], to give six fragments of 67, 115, 461, 530, 1448, and 1889 bp. The 115, 461 and 1448 bp fragments are detected by three digital PCR (dPCR) assays: ADH-115 bp, ADHβ andADHδ respectively.

DNA extraction

In all experiments, cfDNA was extracted from 1 mL CSF using the following kits according to the manufacturers' instructions: QIAamp Circulating Nucleic Acid Kit ('CNA', Qiagen, Hilden, Germany), Quick-cfDNA Serum & Plasma Kit ('QSP', Zymo, Freiburg im Breisgau, Germany), NucleoSnap® DNA Plasma ('NS', Macherey-Nagel, Düren, Germany), Plasma/Serum Circulating DNA Purification Mini Kit (Slurry Format) ('PCP', Norgen, Thorold, Canada). The required ethanol and isopropanol was purchased from Sigma-Aldrich (Gillingham, UK). For the QSP Kit, an optional step to enhance total yield was performed in which the elution buffer is heated to 60–70 °C and passed twice through the elution columns. All samples were eluted in 50 μL Elution Buffer except for the PCP Kit (100 μL, at the recommendation of the manufacturer). In Phase 1, replicate extractions ($n = 3$) were performed for each CSF Pool. In Phase 2, cfDNA was extracted from individual patient CSF samples in single extractions. All CSF cfDNA extracts were stored at 4 °C (immediate use) or − 20 °C (longer-term storage [less than 1 month]). For some CSF samples, CSF was added directly to qPCR without DNA extraction and are referred to as "neat".

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) for the nuclear repetitive element *AluJ* was performed using the "ALUJ" assay as previously described [25,28]. Primer sequences are provided in Supplementary Methods Table 1. All qPCR reactions were performed with 1 × FastEvaGreen qPCR mastermix (Biotium, Fremont, US) in a total volume of 20 μL with 2.5 μL CSF cfDNA extracts or genomic DNA (gDNA) (standard curves). All reactions were performed on a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Loughborough, UK) with cycling conditions given in Supplementary Methods Table 2 and data processed with SDS version 2.4 (Thermo Fisher Scientific). All experiments included no-template controls. The standard curve consisted of a 5-fold dilution series of female human gDNA (Promega, Southampton, UK) from ~ 10 to ~ 0.003 haploid genome equivalents (GE) per reaction in Phase 1 and from ~ 1000 to ~ 0.1 GE per reaction in Phase 2. Duplicate reactions were performed for each point on the standard curve and single reactions for CSF extracts.

Measurements of non-extracted CSF were performed by qPCR using the ALUJ assay (to avoid possible aerosol formation during dPCR droplet formation or reading) using 6.2 μL neat or diluted CSF (prepared by mixing 50 μL CSF with 50 μL nuclease-free water). Further qPCR experiments with non-extracted CSF using the mtND4 assay (as described below) were performed as described in Supplementary methods. Further qPCR information in accordance with the MIQE guidelines [29] is provided in Supplementary MIQE/dMIQE information.

Droplet digital PCR

dPCR assays were based on previous publications: ADH plasmid assays (ADHβ [30], ADHδ [30] and ADH-115 bp [25]); human genomic assays to the nuclear gene *RPPH1* (NCBI Gene ID 85495) [25]; and mitochondrial genomic regions: a 123 bp region of the 12S rRNA gene (*MT-RNR1*, NCBI Gene ID 4549) ('mtDNA-123' assay [18]) and NADH dehydrogenase 4 (*MT-ND4*, NCBI Gene ID 4538) (mtND4 assay [31]). The mtND4 assay used EvaGreen detection chemistry while all other dPCR assays included fluorescent hydrolysis probes. dPCR experiments using the QX200™ Droplet Digital™ PCR system (Bio-Rad, Watford, UK) were performed in accordance with the manufacturer's instructions and dMIQE guidelines [32]. Further details are provided in Supplementary methods and MIQE/dMIQE Information files.

dPCR reactions contained variable template volumes as follows. In Phase 1, to compare equivalent amounts of CSF extracts between kits, a greater volume of PCP kit extracts was added to account for the larger elution volume used compared to the other three extraction kits. For mtDNA-123 and RPPH1 assays, 5 μL template was added for CNA, QSP and NS kits and 9 μL (maximum possible input volume) for PCP. For ADH analysis, 2.5 μL template was added for the CNA, QSP and NS kits and 5 μL for the PCP kit ($n = 1$ dPCR reactions for all three assays). In Phase 2, 5 μL cfDNA template was added per reaction for mtDNA-123 and mtND4 analysis, and 2.5 μL for ADH analysis ($n = 1$ dPCR reactions for all three assays).

In Phase 1, three ADH assays, ADH-115 bp, ADHβ and ADHδ, were used to measure the recovery of the 115-, 461- and 1448-bp ADH plasmid fragments respectively from cfDNA extractions. Percentage yield was calculated by dividing the ADH copy number concentration in the eluates with that measured in the 'spike-only' ADH control material, which had not undergone extraction, within the same dPCR experiment. In Phase 2, ADH plasmid recovery from individual CSF cfDNA extractions was measured with the ADHβ assay only. The 'spike-only' ADH control was measured with multiple reactions ($n = 6$ for Phase 1, $n = 3$ for Phase 2).

Data analysis

Data for Phase 1 and 2 samples are provided in Supplementary Tables S2 and S3. Exogenous ADH spike-in recovery and endogenous target cfDNA concentrations were compared between extraction methods (Phase 1) and disease groups (Phase 2) using Graphpad Prism version 6 (Graphpad Software, San Diego, USA). For Phase 1 data, analysis of ADH fragment size recovery was performed by 2-way ANOVA with post-hoc comparison (Tukey's multiple comparison test) with factors, fragment size and extraction kit (data combined for Pools AD and C). Differences in endogenous cfDNA targets between extraction kits were tested by 2-way ANOVA with factors, extraction kit and CSF pool control or AD). For Phase 2 data, evaluation of ADH recovery (single fragment measured) between patient groups was performed by 1-way ANOVA. Statistical analysis of Phase 2 endogenous target data used log-transformed values, and linear regression of paired data for the mtDNA-123 and mtND4 assays performed. Mitochondrial cfDNA concentrations between biological groups were compared by 1-way ANOVA with Tukey's multiple comparison test. Within-group standard deviations (SD) were significantly different between groups for nuclear target cfDNA concentrations (log2 [ALUJ]) and mitochondrial/nuclear target ratio measurements (log2([mtDNA-123]/[ALUJ]), based on Bartlett's test (GraphPad Prism), $p < 0.01$. Therefore, differences between groups for these datasets were analysed using a non-parametric rank-based test (Kruskal Wallis) with Dunn's multiple comparison test. Analysis of covariance for mitochondrial (mtDNA-123) vs. nuclear

(ALUJ) cfDNA concentrations in Phase 2 groups was performed in R version 3.3.3 with natural log-transformed values. The model consisted of a separate linear relationship between the mitochondrial and nuclear cfDNA concentrations for each subject group, and significance tests for differences in gradient and intercept were performed.

Results

Evaluation of methods for CSF cfDNA extraction and quantification

In Phase 1 of the study (Fig. 1), four commercially available extraction kits were compared using two CSF sample pools, from AD dementia patients and controls. Triplicate extraction replicates were performed with each sample pool and quantities of exogenous and endogenous DNA targets measured by qPCR and dPCR.

To investigate cfDNA extraction recovery and any bias towards longer or shorter DNA fragments associated with each kit, an exogenous spike-in control (ADH plasmid) composed of three fragment sizes was added to each CSF sample prior to DNA extraction. The recovery of the 115-, 461- and 1448-bp ADH plasmid fragments was measured after extraction using three dPCR assays that target sequences present in these fragments (ADH-115 bp, ADHβ and ADHδ, respectively) (Fig. 2 A). As plasmid ADH is an exogenous target, the data are displayed with the replicate extractions from both CSF pools combined for each kit ($n = 6$, Fig. 2A). Overall, the PCP kit produced significantly lower recoveries of all ADH fragments compared to the other three kits tested ($p < 0.001$), with no significant differences between the other three methods. Evaluating the potential bias of kits towards shorter or longer DNA fragments, the CNA kit recovered smaller fragments with slightly higher efficiency (78 %) compared to the 461 and 1448 bp fragments (68 % and 67 % respectively) ($p < 0.001$). The QSP recovered all three fragments with similar efficiency (mean 68 %, $p =$ N.S. for differences between fragments). The PCP kit had a lower extraction yield overall and a bias in recovery towards the larger ADH fragments [1448 bp (25 %) > 461 bp (16 %) > 115 bp (8 %), $p < 0.01$]. Finally, recovery of the 115 bp fragment was 10% lower (69 %) than the 461- and 1448 bp fragments (79 %) ($p < 0.001$) with the NS kit. Evaluating the repeatability associated with replicate extractions for each kit across the three ADH fragments analysed: the CNA and QSP showed comparable % coefficient of variation (CV) values (4–9 %, CNA; 4–8 %, QSP), whilst variability was higher for the NS (%CV 5–19 %) and PCP kits extractions (27–40 %).

To further compare extraction recovery between kits, the yields of endogenous mitochondrial and nuclear genomic targets in cfDNA were measured (Fig. 2B–D). Mean mitochondrial cfDNA concentrations in each CSF pool were in the order of ~ 10^3 GE/mL CSF (Fig. 2B). Yield using the PCP kit was ~ 2-fold lower than the QSP ($p < 0.05$) and NS kits ($p < 0.001$). The CNA kit showed a modest reduction in yield (30 %) compared to the NS kit ($p = 0.03$). Nuclear cfDNA concentration was measured by an assay to *RPPH1* which is present as a single copy per haploid genome (Fig. 2C; results for both CSF pools combined). *RPPH1* was detected in only 50 % of all extracts tested (Fig. 2C) and at < 10 copies per dPCR assay (Supplementary Fig. S1), suggesting that its concentration was below the limit of detection when extract equivalent to 100 μL CSF was measured. In contrast, owing to its high copy number (~ 10^6 repeats per haploid genome), *AluJ* was measured reliably in all extracts from both CSF pools, with between 7 and 58 GE/mL CSF being observed (Fig. 2D), suggesting that the low dPCR input concentration (equivalent to 100 μL CSF; ≤ 5 copies/reaction) as the cause of the sporadic *RPPH1* detection. The recovery of nuclear-derived cfDNA followed a similar pattern to the mtDNA, with the yield 2- to 3-fold lower with the PCP kit compared to the other three methods ($p < 0.001$). The CNA kit showed a slightly lower yield (30 %) than the QSP ($p < 0.001$) and NS kits ($p = 0.01$). The ratio of mitochondrial to nuclear-derived cfDNA is shown in Fig. 1E. No significant differences were found in the mitochondrial/nuclear cfDNA ratio between extraction methods, however a small difference between the two CSF pools was found ($p < 0.05$; 158 (Pool C) vs. mean 113 (Pool AD)).

The impact of neat CSF compared to cfDNA extracted from CSF using an extraction method was assessed on nuclear cfDNA quantification using the ALUJ qPCR assay. Neat CSF resulted in ~ 14-fold lower measured cfDNA concentrations compared to maximum values measured in CSF extracts and inhibition continued to be observed following dilution of neat CSF (Fig. 2D). Although it was not possible to analyse mtDNA targets in neat CSF by dPCR for biosafety reasons (Materials and Methods), qPCR analysis showed that neat and diluted CSF samples also caused inhibition of mitochondrial assay amplification (Supplementary Fig. S2), precluding further direct analysis of mtDNA in CSF samples.

To select the extraction method to be used for Phase 2 of the study, the performance of the four methods tested was reviewed in terms of efficiency of both spiked and endogenous DNA extraction. The PCP method was excluded as it showed reduced yield and a tendency towards recovery of longer DNA fragments. The remaining three methods demonstrated broadly similar performance in cfDNA yield and extraction repeatability (Table S4). In particular, the CNA demonstrated the highest levels of the short ADH spike-in fragment; combined with practical considerations, the CNA method was selected for further CSF cfDNA extractions.

Comparison of mitochondrial and nuclear cfDNA in AD dementia, brain cancer and control subjects

In Phase 2 of the study (Fig. 1), cfDNA was extracted using the CNA method from individual CSF samples from patients with AD dementia ($n = 12$), primary or metastatic brain tumours ($n = 12$) and controls ($n = 10$) to illustrate the application of the methodology to the analysis of putative cfDNA biomarkers and evaluate the magnitude of biological variation within and between groups.

To monitor extraction efficiency, each sample was spiked with the fragmented ADH plasmid prior to DNA extraction. Extraction yield was quantified with the ADHβ assay (targeting the 461 bp fragment) and was in a similar range to that observed in the analysis of the CSF pools (81–86 %, Fig. 3 A), except for one sample from the AD group (AD7) which had low yield (46 %) and was excluded from further analysis. No significant differences in extraction efficiency were observed between the three groups; however, the variability within the cancer group (5 % CV) was lower than that in the control and AD groups (12 % and 13 % CV respectively).

To investigate PCR assay as a potential source of bias in the quantification of mitochondrial cfDNA, CSF extracts were quantified by dPCR with assays to two mitochondrial genomic targets using alternative reaction chemistries: an intergenic region (mtDNA-123 hydrolysis probe assay used in Phase 1) and *ND4* (mtND4 intercalating dye assay). Linear regression analysis identified one sample from the AD group (AD3) as an outlier (Supplementary Fig. S4), which is likely to originate from a technical error affecting one of the measurements. Following exclusion of this datapoint, high concordance was demonstrated between the two assays, with a best-fit slope of 1.02 (95 % confidence interval: 0.9812–1.060) and $R^2 = 0.9893$ (Fig. 3B).

Mitochondrial cfDNA concentrations from individual CSF samples in the control and two disease groups are presented in Fig. 3C (mtDNA-123 assay) and Fig. S3 (mtND4 assay). Log-transformation was necessary for Phase 2 results due to the higher concentration and variability of the brain cancer group cfDNA concentration data compared to that of the control and AD groups. Log-transformed mean mitochondrial cfDNA levels were similar in control (3.0×10^3 GE/mL CSF) and AD groups (2.4×10^3 GE/mL CSF) ($p =$ N.S.), but were an order of magnitude higher in the CSF of brain cancer patients (3.3×10^4 GE/mL CSF, range 9.8×10^2–2.4×10^5) ($p < 0.001$).

Analysis of nuclear genome-derived cfDNA in the patient and control samples with the ALUJ assay revealed a similar trend to the

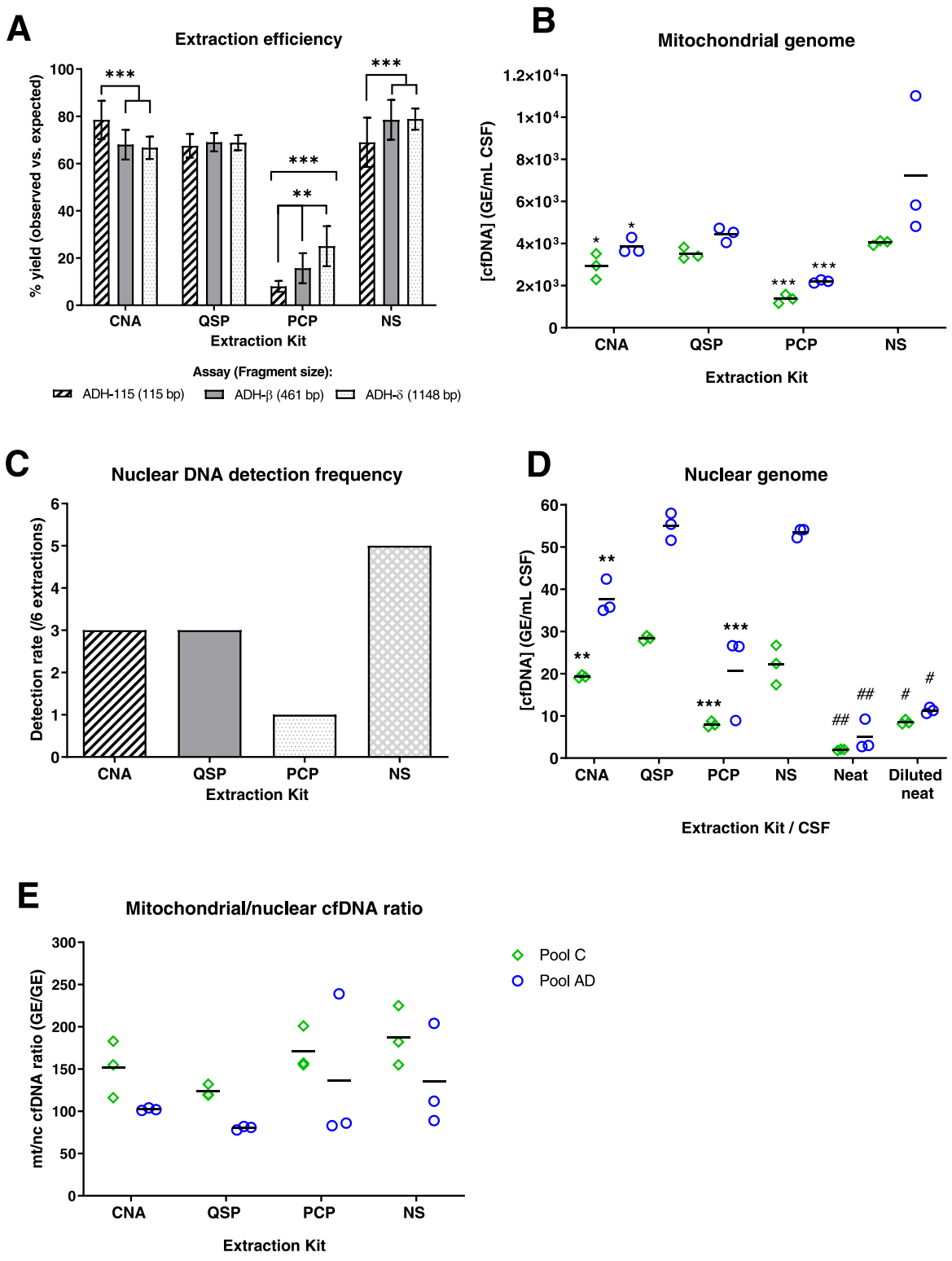

Fig. 2. Assessment of extraction efficiency and fragment size bias (A) and CSF cfDNA yield (B–D) of four extraction kits (CNA, QSP, PCP, and NS). (A) Mean extraction efficiencies of the CNA, QSP, PCP, and NS kits are expressed as a percentage of input (10^6 copies per millilitre of CSF) ± standard deviation ($n = 6$ extractions, CSF Pool 'C' and Pool 'AD' data combined) for 115-, 461- and 1448-bp fragments of the ADH plasmid spike-in. (B) Mitochondrial cfDNA yield values from replicate extractions ($n = 3$) for CSF Pool 'C' (green diamonds) and CSF Pool 'AD' (blue circles) quantified with the mtDNA-123 assay. * $p \leq 0.05$ CNA < NS; ***$p \leq 0.001$ PCP < NS. (C) Detection rate of nuclear single copy genomic locus DNA measured by the *RPPH1* assay with dPCR for both CSF Pool 'C' and Pool 'AD' combined. (D) Nuclear cfDNA yield values from replicate extractions ($n = 3$) for each CSF pool (symbols as part B) quantified with the ALUJ assay (qPCR). **$p \leq 0.01$, CNA < QSP, NS kits. *** $p \leq 0.001$, PCP < CNA, QSP, NS. Neat CSF and diluted neat CSF (neat CSF diluted 1:1 with nuclease free water) were quantified in addition to extracts. ##$p \leq 0.001$ (Neat vs. all extraction kits). #$p \leq 0.001$ (Diluted neat vs. CNA and QSP kits). (E) Ratio of mitochondrial/nuclear cfDNA in each CSF pool (symbols as part B) (mt-123 and ALUJ assays). GE = Genome Equivalents. Mean results are shown as horizontal bars.

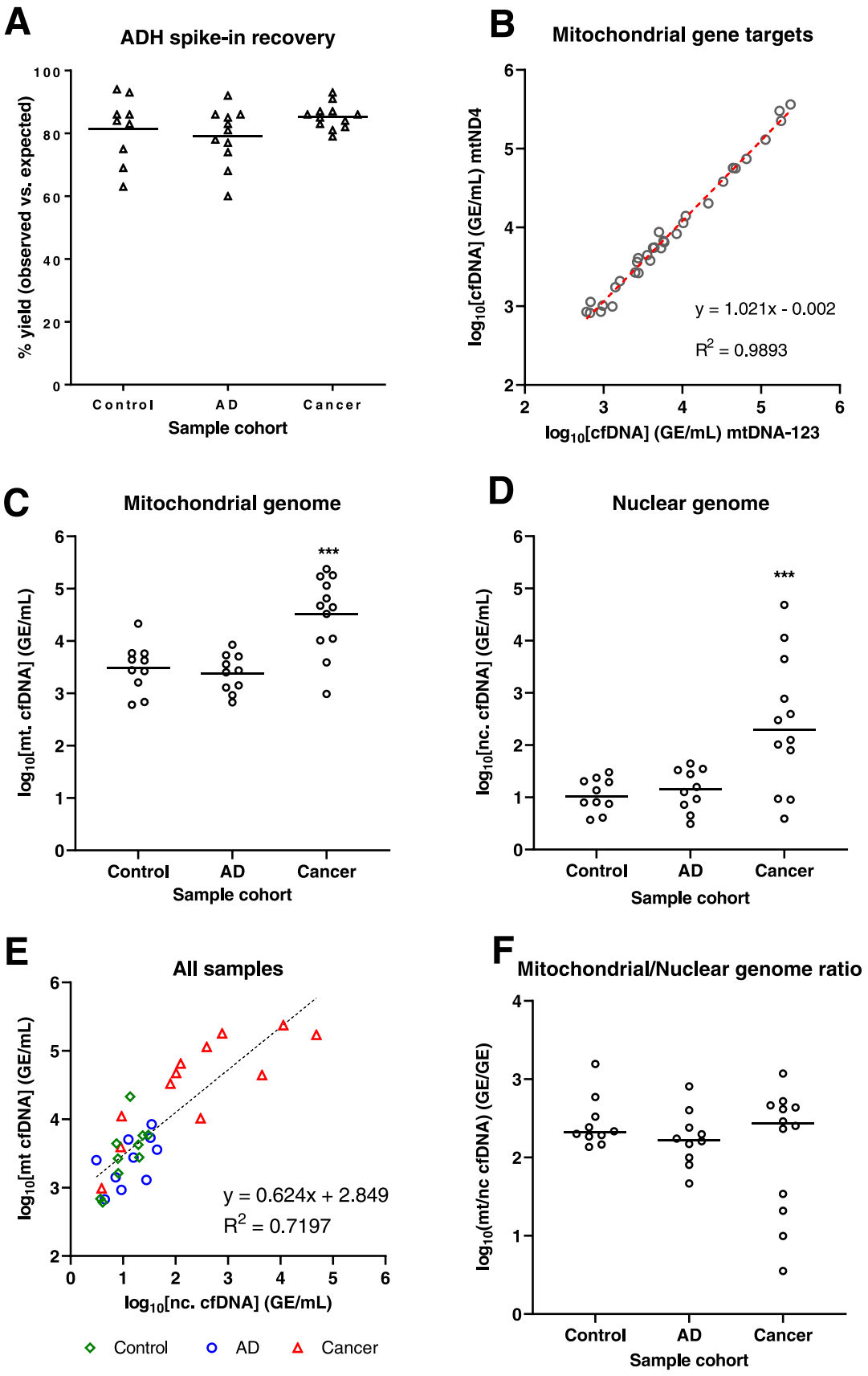

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Fig. 3. Analysis of individual CSF sample extracts (Phase 2 analysis) from three groups: To reflect the fact that controls are not classed as patients, only the AD dementia and brain cancer groups. (A) Extraction efficiency showing ADH plasmid spike-in recovery in each sample as a percentage of input amount (10^6 copies per mL CSF) measured by dPCR. (B) Linear regression analysis mtDNA concentration in samples from all three groups measured by alternative assays (mtDNA-123 vs. mtND4). (C, D) Analysis of mitochondrial (C) and nuclear (D) cfDNA levels in individual CSF samples. Samples were measured by dPCR with the mtDNA-123 assay (C) and by qPCR with the ALUJ assay (D). (E) Linear regression analysis of all Phase 2 data ($n = 32$) showing the correlation between mitochondrial and nuclear cfDNA in CSF. (F) Normalised mitochondrial cfDNA concentration in three groups: controls ($n = 10$), AD ($n = 12$) and cancer patients ($n = 12$). Normalisation was performed by calculating the ratio of mitochondrial to nuclear genomic copies for each sample. Symbols show individual samples; horizontal bars: mean (A, C) or median (D, F) values according to whether the dataset was analysed using parametric or non-parametric methods. *** $p \leq 0.001$ (vs. control group). *mt*, mitochondrial; *nc*, nuclear.

mitochondrial cfDNA results (Fig. 3D). Comparable levels of nuclear cfDNA were found between the control and AD groups (median of log-transformed values, 10 and 14 GE/mL CSF respectively; p = N.S.) and elevated levels in the cancer group (194 GE/mL CSF) compared to each of the other two groups ($p < 0.001$). The inter-sample variability within the brain cancer group was also considerably higher (SD ± 18- and 5-fold of the mean for nuclear and mitochondrial targets respectively) than within the control or the AD group (SD ± 2-fold of the mean) (Supplementary Table S4).

Normalisation approach for measurement of mitochondrial cfDNA biomarker concentration

To investigate further the relationship between mitochondrial and nuclear cfDNA levels in CSF under different biological conditions, individual mtDNA concentrations were compared to the corresponding nuclear cfDNA concentrations (Fig. 3E). Linear regression analysis of mtDNA-123 vs. ALUJ assay data, revealed a significant correlation between mitochondrial and nuclear CSF cfDNA copy number concentrations for pooled data from the three groups ($R^2 > 0.7$, $p < 0.0001$). It was noted that higher cfDNA concentrations observed in the brain cancer group may have had a dominant leveraging effect on this regression analysis. This was confirmed by analysis of covariance where a steeper gradient was observed for the cancer group compared to the control group ($p = 0.052$) (Table S5). The observed proportionality between the amount of nuclear and mitochondrial cfDNA in CSF suggested that calculation of the ratio of mitochondrial cfDNA concentration to nuclear cfDNA concentration may be a valid normalisation approach for measuring mtDNA. Therefore mt/nc cfDNA ratios were calculated for all three groups and were found to be of the same order of magnitude (median 208, 168 and 270 mt cfDNA/nc cfDNA (GE/GE) for control, AD and cancer groups respectively; based on analysis of log-transformed values) with no significant differences between groups ($p = 0.56$) (Fig. 3F).

Discussion

CSF is a source of potential cfDNA biomarkers in a number of fields, including neurology, virology, oncology and intensive care medicine [33–36]. In oncology, cfDNA targets are normally tumour mutations present in the nuclear genome, whereas mitochondrial targets are candidate biomarkers for diseases associated with cellular dysfunction such as AD. There has been significant progress in standardisation of sample processing and analysis protocols for circulating cfDNA, including publication of ISO guidelines [37] developed through projects such as Cancer-ID [22] and SPIDIA [23]. In addition, matrix-based circulating tumour DNA QC materials are being validated in a three phase study led by the FNIH Biomarkers consortium [38,39] and reporting recommendations for pre-analytical blood processing variables developed by the BloodPAC consortium [40,41]. Likewise, consensus guidelines for pre-analytical processing of CSF are widely followed for proteins biomarkers in neurodegenerative diseases [42]. These are in stark contrast to the current status in cfDNA analysis in CSF, where validated pre-analytical approaches and controls are not available. Therefore, the aim in this study was to evaluate sample processing approaches for cfDNA extraction and compare assays for the quantification of various cfDNA targets, in order to investigate the impact of these on biomarker analysis.

In Phase 1 of the study, four cfDNA extraction methods were evaluated and compared with direct input of neat CSF into downstream reactions. Recovery of both cfDNA and a spike-in control material varied between the four methods tested, with one kit showing sub-optimal performance and 2- to 3-fold lower concentration values than the other three approaches. The recovery of an exogenous spike-in [25] provided a useful metric of extraction efficiency. Although 'naked DNA' may not behave exactly the same as biological cfDNA complexed with proteins such as nucleosomes [43], which are acted on by proteinase and chaotropic agents in lysis buffers, the use of spike-in material to reflect aspects such as affinity of the extraction method's DNA adsorption matrix for similar sized DNA and carryover of inhibitors, can add confidence to method validation. In this study, the dual approach of measuring both exogenous and endogenous DNA indicated that the majority of cfDNA was recovered during extraction with the three "better-performing" kits and underscores the importance of validating nucleic acid extraction for cfDNA from different biological matrices.

Although it has been raised that silica purification columns are less effective in the extraction of mtDNA compared to nuclear cfDNA, leading to distortion in the mt/nc ratios [44], this finding may be attributable to the use of RNase treatment in the commercial protocols applied in an earlier study and its effect on degradation of mitochondrial DNA-RNA hybrid structures ([44] and references therein). The methods evaluated in the present study did not utilise RNase treatment. A recent study found comparable concentrations of mitochondrial and nuclear-derived cfDNA in diluted CSF vs. extracted CSF and provides evidence that the majority of mitochondrial cfDNA is retrieved during extraction [45]. In addition, no differences in mitochondrial to nuclear cfDNA ratios were observed between the methods tested. Use of non-purified samples in molecular analyses raises biosafety issues as well as leading to inhibition of reaction kinetics, as demonstrated in this study. Although, reportedly, dPCR is more resilient to reaction inhibitors than qPCR, assays should be tested on an individual basis to confirm this [46]. Moreover, cfDNA extraction may enable the recovery of mtDNA present in extracellular vesicles in biofluids [47] or cell-free intact mitochondria [48] which may not be detected in unpurified CSF samples, as well as concentrating samples, thus improving analytical sensitivity.

The results of measuring nuclear genome-derived cfDNA targets vary markedly between previous reports in the neurology and oncology fields; in neurodegenerative disease studies [e.g. [18]], samples containing > 1 GE per reaction are commonly excluded, which is in contrast to studies involving brain tumours where cancer genetic biomarkers are detected in CSF [e.g. [20]]. The concentration of nuclear genome-derived cfDNA observed in the current study for control subjects and AD patients was < 100 nuclear GE/mL, compared to samples of circulating cfDNA, which typically contain at least 1000 nuclear GE/mL plasma (~ 3 ng/mL) in healthy donors [25]. This low concentration resulted in sporadic detection of a target present at a single copy per haploid genome, suggesting that the sample exclusion criterion of > 1 GE per ~ 10 μL CSF) [18] may be influenced by random sampling error. In contrast, cfDNA in the 12 CSF samples from primary and secondary brain cancer patients was elevated to concentrations comparable to circulating cfDNA which may be due to inflammation and metastasis

[49]. Taken together, these results lead to the conclusion that single copy nuclear loci are not appropriate for robust quantification of nuclear-derived cfDNA in CSF of non-cancer patients. Furthermore, the greater sensitivity afforded by an assay corresponding to repetitive elements, such as the ALUJ assay used in this study, provides more robust and precise measurements.

Other aspects of CSF sample processing which are worthy of future investigation include controlling for cellular contamination associated with lumbar puncture or lysis of lymphocytes in CSF. The total DNA content of CSF samples was observed to be lower following centrifugation of the samples, indicating that genomic DNA from cells may contaminate the true cfDNA [20]. Therefore, standardised protocols for CSF sample collection, processing and storage are warranted. For circulating cfDNA analysis, plasma samples normally undergo a second centrifugation step (after the first plasma separation) at 4 °C to remove residual cellular material [37], therefore it is recommended that a standardised centrifugation process is followed for CSF cfDNA analysis, depending on whether exosomes are separated from cfDNA upon centrifugation [47]. It may be possible to improve lumbar collection procedures to reduce the risk of cell contamination. Furthermore, the choice of collection tube may be relevant, with some CSF cfDNA studies using EDTA preservation tubes [50] which may inhibit nucleases in the sample [51]. Proteins that tend to form aggregates, e.g. Aβ1–42, have the propensity to become adsorbed onto certain tube materials, such as glass or polystyrene, resulting in false-positive results in AD diagnostics. The use of polypropylene tubes is therefore recommended for protein markers [52] and this recommendation may also apply to cfDNA in CSF. It should also be noted that the sample containers selected should be made of the same material, at least within one centre, from lumbar puncture to laboratory analysis (including for aliquoting, biobanking, etc.) [53]. Freeze-thawing of CSF samples and purified cfDNA is another important pre-analytical consideration. It was not possible to fully normalise CSF freeze-thawing in the three cohorts investigated in this study: individual control and AD samples were analysed following two freeze-thaw cycles compared to a single cycle for the cancer samples. These are within the range (three freeze-thaw cycles) where mitochondrial cfDNA has been shown to be stable by other investigators [54].

Mitochondrial cfDNA concentration is a candidate biomarker in a number of fields (summarised in [48]). A systematic review of mtDNA as a biomarker for critically ill patients observed issues with “utiliz[ing] different protocols to measure mtDNA” and the need for “standardisation of mtDNA protocols” for clinical translation [36]. Methodological parameters have also been raised as confounding factors in the evaluation of cell-free mtDNA as a biomarker in neurological disorders [19]. In contrast to some previous reports [18,55], mitochondrial cfDNA levels were not found to be different between AD dementia patients and controls in the results presented here, which is consistent with a recent study in post-mortem CSF samples [48]; however, conclusions from the current study are limited due to the small number of cases/controls, which are also not age-matched (Table S1). Other studies have found raised mitochondrial cfDNA in AD patients but with high inter-individual variability, reducing its sensitivity as a diagnostic biomarker [54]. Monitoring extraction efficiency and measuring both mitochondrial and nuclear-derived cfDNA, enabled QC of the pre-analytical process and investigation of both sources of cfDNA, as opposed to potential exclusion of samples with nuclear-derived cfDNA. As well as being utilised as a technical control, the normalisation of mitochondrial cfDNA levels to nuclear GE may be useful as an indicator of mitochondrial ratio in the cells which contribute to the cfDNA through apoptosis or necrosis. Due to the limitations noted, further studies would be required to apply these methodological approaches to a full diagnostic evaluation of mitochondrial cfDNA levels in AD or other neurodegenerative diseases.

The application of widely used technologies such as dPCR, NGS, and methylated DNA immunoprecipitation for CSF biopsy analysis to detect mutations, copy number variations and aberrant methylation, is an effective approach for the diagnosis and subtyping of brain tumours. Alongside neuroimaging and tissue biopsy, CSF biopsy can be utilised to profile tumour stage, monitor progression, and select the optimal treatment plan [56].

While cell-free nucleic acid biomarkers in CSF continue to be applied to diagnosis of CNS cancers [35,57], blood-borne nucleic acids have been the focus of some recent research into novel AD biomarkers: in one study, the concentration of total cfDNA was found to be raised in AD [58] and, a second study found cell-free mRNAs differentially expressed between AD and healthy controls [59]. A recent meta-analysis highlighted differentially expressed miRNA in blood, rather than CSF [60]. Methylation patterns of cfDNA have been shown to indicate tissue of origin, and despite potential limitations of the blood brain barrier, methylation signatures of neural cell-types were detected in multiple sclerosis and traumatic brain injury [61], indicating that methylation cfDNA biomarkers may be a route to screen for neurodegenerative disorders as it is being applied for cancer early detection [62].

In summary, the potential of cell-free nucleic acids in CSF as diagnostic analytes is still being realised for CNS cancers [63,64] and neurological disorders [65]. Validation of analytical and pre-analytical factors supports robust clinical validation of candidate biomarkers, therefore the approaches for evaluation and QC of cfDNA extraction efficiency and measurement of total cfDNA outlined in this study will hopefully support future clinical implementation of CSF for liquid biopsy and early disease detection.

CRediT authorship contribution statement

Petros Takousi: Project administration, Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Alison S. Devonshire:** Visualization, Conceptualization, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Nicholas Redshaw:** Methodology, Investigation, Writing – review & editing. **Louisa von Baumgarten:** Project administration, Conceptualization, Resources, Supervision, Writing – review & editing. **Alexandra S. Whale:** Methodology, Investigation, Writing – review & editing. **Gerwyn M. Jones:** Investigation. **Ana Fernandez-Gonzalez:** Investigation, Writing – review & editing. **Jan Martin:** Resources, Writing – review and editing. **Carole A. Foy:** Project administration, Writing – review & editing. **Panagiotis Alexopoulos:** Project administration, Conceptualization, Resources, Supervision, Writing – review & editing. **Jim F. Huggett:** Project administration, Conceptualization, Supervision, Writing – review & editing. **Robert Perneczky:** Project administration, Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Robert Perneczky reports a relationship with Janssen that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Biogen that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Takeda that includes: funding grants. Robert Perneczky reports a relationship with Schwabe that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Grifols that includes: consulting or advisory and speaking and lecture

fees. Robert Perneczky reports a relationship with Roche that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Eli Lilly that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Bayer that includes: consulting or advisory and speaking and lecture fees. Robert Perneczky reports a relationship with Novo Nordisk that includes: consulting or advisory and speaking and lecture fees. Alison Devonshire reports a relationship with Roche that includes: paid expert testimony.

Data Availability

Sample data is available in Supplementary Tables.

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AD received paid expert testimony from Roche.

Availability of data and materials

Sample data is available in Tables S2 and S3. Additional data available upon request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.10.001.

References

[1] Lo YM. Circulating nucleic acids in plasma and serum: an overview. Ann N Y Acad Sci 2001;945:1–7. https://doi.org/10.1111/j.1749-6632.2001.tb03858.x.
[2] Ziegler A, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine. Cancer Treat Rev 2002;28(5):255–71. https://doi.org/10.1016/s0305-7372(02)00077-4.
[3] Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. Mutat Res 2007;635(2–3):105–17. https://doi.org/10.1016/j.mrrev.2006.11.002.
[4] Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest 1966;45(11):1732–40. https://doi.org/10.1172/JCI105479.
[5] Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977;37(3):646–50. ⟨https://www.ncbi.nlm.nih.gov/pubmed/837366⟩.
[6] Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer 1983; 51(11):2116–20. https://doi.org/10.1002/1097-0142(19830601)51:11<2116::aid-cncr2820511127>3.0.co;2-s.
[7] Stroun M, Anker P, Lyautey J, Lederrey C, Maurice PA. Isolation and characterization of DNA from the plasma of cancer patients. Eur J Cancer Clin Oncol 1987;23(6):707–12. https://doi.org/10.1016/0277-5379(87)90266-5.
[8] Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 1989; 46(5):318–22. https://doi.org/10.1159/000226740.
[9] Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994; 86(4):774–9. https://doi.org/10.1111/j.1365-2141.1994.tb04828.x.
[10] Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomark Prev 1994;3(1):67–71. ⟨https://www.ncbi.nlm.nih.gov/pubmed/8118388⟩.
[11] Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2(9):1033–5. https://doi.org/10.1038/nm0996-1033.
[12] Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350(9076):485–7. https://doi.org/10.1016/S0140-6736(97)02174-0.
[13] Butt AN, Swaminathan R. Overview of circulating nucleic acids in plasma/serum. Ann N Y Acad Sci 2008;1137:236–42. https://doi.org/10.1196/annals.1448.002.
[14] Swinkels DW, de Kok JB, Hanselaar A, Lamers K, Boerman RH. Early detection of leptomeningeal metastasis by PCR examination of tumor-derived K-ras DNA in cerebrospinal fluid. Clin Chem 2000;46(1):132–3. ⟨https://www.ncbi.nlm.nih.gov/pubmed/10620587⟩.
[15] Angert RM, Leshane ES, Yarnell RW, Johnson KL, Bianchi DW. Cell-free fetal DNA in the cerebrospinal fluid of women during the peripartum period. Am J Obstet Gynecol 2004;190(4):1087–90. https://doi.org/10.1016/j.ajog.2003.10.562.
[16] Shi W, Lv C, Qi J, Zhao W, Wu X, Jing R, et al. Prognostic value of free DNA quantification in serum and cerebrospinal fluid in glioma patients. J Mol Neurosci 2012;46(3):470–5. https://doi.org/10.1007/s12031-011-9617-0.
[17] Wang HC, Yang TM, Lin WC, Lin YJ, Tsai NW, Liou CW, et al. The value of serial plasma and cerebrospinal fluid nuclear and mitochondrial deoxyribonucleic acid levels in aneurysmal subarachnoid hemorrhage. J Neurosurg 2013;118(1):13–9. https://doi.org/10.3171/2012.8.jns112093.
[18] Podlesniy P, Figueiro-Silva J, Llado A, Antonell A, Sanchez-Valle R, Alcolea D, et al. Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease. Ann Neurol 2013;74(5):655–68. https://doi.org/10.1002/ana.23955.
[19] Sondheimer N, Zollo O, Van Deerlin V, Trojanowski JQ. Analysis of cerebrospinal fluid mitochondrial DNA levels in Alzheimer disease. Ann Neurol 2014;75(3): 458–60. https://doi.org/10.1002/ana.24107.
[20] Pan W, Gu W, Nagpal S, Gephart MH, Quake SR. Brain tumor mutations detected in cerebral spinal fluid. Clin Chem 2015;61(3):514–22. https://doi.org/10.1373/clinchem.2014.235457.
[21] Hulstaert F, Blennow K, Ivanoiu A, Schoonderwaldt HC, Riemenschneider M, De Deyn PP, et al. Improved discrimination of AD patients using beta-amyloid(1–42) and tau levels in CSF. Neurology 1999;52(8):1555–62. https://doi.org/10.1212/wnl.52.8.1555.
[22] CANCER-ID project website; Available from: ⟨https://www.cancer-id.eu/⟩. [Accessed 31 January 2022].
[23] SPIDIA/SPIDIA4P project website; Available from: ⟨https://www.spidia.eu⟩. [Accessed 31 January 2022].
[24] Lampignano R, Neumann MHD, Weber S, Kloten V, Herdean A, Voss T, et al. Multicenter evaluation of circulating cell-free DNA extraction and downstream analyses for the development of standardized (pre)analytical work flows. Clin Chem 2020;66(1):149–60. https://doi.org/10.1373/clinchem.2019.306837.
[25] Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Anal Bioanal Chem 2014;406 (26):6499–512. https://doi.org/10.1007/s00216-014-7835-3.
[26] Vandermeeren M, Mercken M, Vanmechelen E, Six J, van de Voorde A, Martin JJ, et al. Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay. J Neurochem 1993;61(5):1828–34. https://doi.org/10.1111/j.1471-4159.1993.tb09823.x.
[27] Vanderstichele H, Van Kerschaver E, Hesse C, Davidsson P, Buyse MA, Andreasen N, et al. Standardization of measurement of beta-amyloid(1–42) in cerebrospinal fluid and plasma. Amyloid 2000;7(4):245–58. https://doi.org/10.3109/13506120009146438.
[28] Cannas A, Kalunga G, Green C, Calvo L, Katemangwe P, Reither K, et al. Implications of storing urinary DNA from different populations for molecular analyses. PLoS One 2009;4(9):e6985. https://doi.org/10.1371/journal.pone.0006985.
[29] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22. https://doi.org/10.1373/clinchem.2008.112797.
[30] Sanders R, Huggett JF, Bushell CA, Cowen S, Scott DJ, Foy CA. Evaluation of digital PCR for absolute DNA quantification. Anal Chem 2011;83(17):6474–84. https://doi.org/10.1021/ac103230c.
[31] Belmonte FR, Martin JL, Frescura K, Damas J, Pereira F, Tarnopolsky MA, et al. Digital PCR methods improve detection sensitivity and measurement precision of low abundance mtDNA deletions. Sci Rep 2016;6:25186. https://doi.org/10.1038/srep25186.
[32] Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin Chem 2013;59(6):892–902. https://doi.org/10.1373/clinchem.2013.206375.
[33] Gambardella S, Limanaqi F, Ferese R, Biagioni F, Campopiano R, Centonze D, et al. ccf-mtDNA as a potential link between the brain and immune system in neuro-immunological disorders. Front Immunol 2019;10:1064. https://doi.org/10.3389/fimmu.2019.01064.
[34] Spudich S, Robertson KR, Bosch RJ, Gandhi RT, Cyktor JC, Mar H, et al. Persistent HIV-infected cells in cerebrospinal fluid are associated with poorer neurocognitive performance. J Clin Invest 2019;129(8):3339–46. https://doi.org/10.1172/jci127413.
[35] Zorofchian S, Iqbal F, Rao M, Aung PP, Esquenazi Y, Ballester LY. Circulating tumour DNA, microRNA and metabolites in cerebrospinal fluid as biomarkers for central nervous system malignancies. J Clin Pathol 2019;72(4):271–80. https://doi.org/10.1136/jclinpath-2018-205414.
[36] Harrington JS, Huh J-W, Schenck EJ, Nakahira K, Siempos II, Choi AMK. Circulating mitochondrial DNA as predictor of mortality in critically Ill patients: a

systematic review of clinical studies. Chest 2019;156:1120–36. https://doi.org/10.1016/j.chest.2019.07.014.
[37] ISO 20186-3: 2019. Molecular in vitro diagnostic examinations — specifications for preexamination processes for venous whole blood — isolated circulating cell free DNA from plasma, ⟨https://www.iso.org/standard/69800.html⟩
[38] Biomarkers Consortium – ctDNA Quality Control Materials Project; Available from: ⟨https://fnih.org/our-programs/biomarkers-consortium/programs/ctdna-reference-materials⟩. [Accessed 31 January 2022].
[39] Williams PM, Forbes T, Lund SP, Cole KD, He H-J, Karlovich C, et al. Validation of ctDNA quality control materials through a precompetitive collaboration of the foundation for the National Institutes of Health. JCO Precis Oncol 2021;5:910–20. https://doi.org/10.1200/po.20.00528.
[40] Blood Profiling Atlas in Cancer (BloodPAC) Consortium website; Available from: ⟨https://www.bloodpac.org/⟩. [Accessed 31 January 2022].
[41] Febbo PG, Martin A-M, Scher HI, Barrett JC, Beaver JA, Beresford PJ, et al. Minimum technical data elements for liquid biopsy data submitted to public databases. Clin Pharm Ther 2020;107(4):730–4. https://doi.org/10.1002/cpt.1747.
[42] del Campo M, Mollenhauer B, Bertolotto A, Engelborghs S, Hampel H, Simonsen AH, et al. Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. Biomark Med 2012;6(4):419–30. https://doi.org/10.2217/bmm.12.46.
[43] Pös O, Biró O, Szemes T, Nagy B. Circulating cell-free nucleic acids: characteristics and applications. Eur J Hum Genet 2018;26(7):937–45. https://doi.org/10.1038/s41431-018-0132-4.
[44] Guo W, Jiang L, Bhasin S, Khan SM, Swerdlow RH. DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. Mitochondrion 2009;9(4):261–5. https://doi.org/10.1016/j.mito.2009.03.003.
[45] Chen A, Li J, Wang L, Huang Q, Zhu J, Wen S, et al. Comparison of paired cerebrospinal fluid and serum cell-free mitochondrial and nuclear DNA with copy number and fragment length. J Clin Lab Anal 2020;34(6):e23238. https://doi.org/10.1002/jcla.23238.
[46] Nixon G, Garson JA, Grant P, Nastouli E, Foy CA, Huggett JF. A comparative study of sensitivity, linearity and resistance to inhibition of digital and non-digital PCR and LAMP assays for quantification of human cytomegalovirus. Anal Chem 2014;86(9):4387–94. https://doi.org/10.1021/ac500208w.
[47] Ye W, Tang X, Liu C, Wen C, Li W, Lyu J. Accurate quantitation of circulating cell-free mitochondrial DNA in plasma by droplet digital PCR. Anal Bioanal Chem 2017;409(10):2727–35. https://doi.org/10.1007/s00216-017-0217-x.
[48] Lowes H, Kurzawa-Akanbi M, Pyle A, Hudson G. Post-mortem ventricular cerebrospinal fluid cell-free-mtDNA in neurodegenerative disease. Sci Rep 2020;10(1):15253. https://doi.org/10.1038/s41598-020-72190-5.
[49] Deisenhammer FBA, Egg R, Gilhus N, Giovannoni G, Rauer S, et al. Routine cerebrospinal fluid (CSF) analysis. In: Gilhus NEBM, Brainin M, (eds.), European handbook of neurologic management. New York: John Wiley and Sons; 2011, p. 5–17.
[50] Meier L, Carlson R, Neßler J, Tipold A. Stability of canine and feline cerebrospinal fluid samples regarding total cell count and cell populations stored in "TransFix®/EDTA CSF sample storage tubes". BMC Vet Res 2020;16(1):487. https://doi.org/10.1186/s12917-020-02698-5.
[51] Barra GB, Santa Rita TH, Vasques JdA, Chianca CF, Nery LFA, Costa SSS. EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. Clin Biochem 2015;48(15):976–81. https://doi.org/10.1016/j.clinbiochem.2015.02.014.
[52] Vanderstichele HM, Janelidze S, Demeyer L, Coart E, Stoops E, Herbst V, et al. Optimized standard operating procedures for the analysis of cerebrospinal fluid Aβ42 and the ratios of Aβ isoforms using low protein binding tubes. J Alzheimers Dis 2016;53(3):1121–32. https://doi.org/10.3233/jad-160286.
[53] Tumani H, Petereit HF, Gerritzen A, Gross CC, Huss A, Isenmann S, et al. S1 guidelines "lumbar puncture and cerebrospinal fluid analysis" (abridged and translated version). Neurol Res Pract 2020;2:8. https://doi.org/10.1186/s42466-020-0051-z.
[54] Cervera-Carles L, Alcolea D, Estanga A, Ecay-Torres M, Izagirre A, Clerigue M, et al. Cerebrospinal fluid mitochondrial DNA in the Alzheimer's disease continuum. Neurobiol Aging 2017;53:192.e1–4. https://doi.org/10.1016/j.neurobiolaging.2016.12.009.
[55] Podlesniy P, Llorens F, Golanska E, Sikorska B, Liberski P, Zerr I, et al. Mitochondrial DNA differentiates Alzheimer's disease from Creutzfeldt-Jakob disease. Alzheimers Dement 2016;12(5):546–55. https://doi.org/10.1016/j.jalz.2015.12.011.
[56] An Y, Fan F, Jiang X, Sun K. Recent advances in liquid biopsy of brain cancers. Front Genet 2021;12:720270. https://doi.org/10.3389/fgene.2021.720270.
[57] von Baumgarten L, Kumbrink J, Jung A, Reischer A, Flach M, Liebmann S, et al. Therapeutic management of neuro-oncologic patients – potential relevance of CSF liquid biopsy. Theranostics 2020;10(2):856–66. https://doi.org/10.7150/thno.36884.
[58] Feger D, Nidadavolu L, Oh E, Abadir P, Gross A. Circulating cell-free DNA is associated with cognitive outcomes. Innov Aging 2020;Suppl._1(4):518. https://doi.org/10.1093/geroni/igaa057.1671.
[59] Toden S, Zhuang J, Acosta AD, Karns AP, Salathia NS, Brewer JB, et al. Noninvasive characterization of Alzheimer's disease by circulating, cell-free messenger RNA next-generation sequencing. Sci Adv 2020;6(50). https://doi.org/10.1126/sciadv.abb1654.
[60] Takousis P, Sadlon A, Schulz J, Wohlers I, Dobricic V, Middleton L, et al. Differential expression of microRNAs in Alzheimer's disease brain, blood, and cerebrospinal fluid. Alzheimers Dement 2019;15(11):1468–77. https://doi.org/10.1016/j.jalz.2019.06.4952.
[61] Lehmann-Werman R, Neiman D, Zemmour H, Moss J, Magenheim J, Vaknin-Dembinsky A, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. Proc Natl Acad Sci USA 2016;113(13):E1826–34. https://doi.org/10.1073/pnas.1519286113.
[62] Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Liu MC, et al. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. Ann Oncol 2020;31(6):745–59. https://doi.org/10.1016/j.annonc.2020.02.011.
[63] Escudero L, Martínez-Ricarte F, Seoane J. ctDNA-based liquid biopsy of cerebrospinal fluid in brain cancer. Cancers 2021;13(9). https://doi.org/10.3390/cancers13091989.
[64] McEwen AE, Leary SES, Lockwood CM. Beyond the blood: CSF-derived cfDNA for diagnosis and characterization of CNS tumors. Front Cell Dev Biol 2020;8(45). https://doi.org/10.3389/fcell.2020.00045.
[65] Kumar S, Reddy PH. Are circulating microRNAs peripheral biomarkers for Alzheimer's disease. Biochim Biophys Acta 2016;1862(9):1617–27. https://doi.org/10.1016/j.bbadis.2016.06.001.