Received: 28 May 2023 | Revised: 11 December 2023 | Accepted: 16 January 2024

DOI: 10.1002/dad2.12555

Alzheimer's & Dementia
Diagnosis, Assessment & Disease Monitoring

SHORT REPORT

Assessment of circulating apoE4 levels from dried blood spot samples in a large survey setting

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Funding information
European Union's Horizon 2020 research and innovation programme, Grant/Award Number: ERC-STG-716065; NIA, Grant/Award Numbers: R01_AG063944, R01_AG056329, U01_AG09740-13S2, P01_AG005842, P01_AG08291, P30_AG12815, R21_AG025169, Y1-AG-4553-01, IAG_BSR06-11, OGHA_04-064, HHSN271201300071C, RAG052527A; Max Planck Society; European Commission, Grant/Award Numbers: QLK6-CT-2001-00360, SHARE-I3: RII-CT-2006-062193, COMPARE: CIT5-CT-2005-028857, SHARELIFE: CIT4-CT-2006-028812, SHARE-PREP: GA N°211909, SHARE-LEAP: GA N°227822, SHARE M4: GA N°261982, DASISH: GA N°283646; Horizon 2020, Grant/Award Numbers: SHARE-DEV3: GA N°676536,

Abstract

INTRODUCTION: The apolipoprotein E (*APOE*) ε4 allele is associated with high risk for Alzheimer's disease. It is unclear whether individual levels of the circulating apoE4 protein in ε4 carriers confer additional risk. Measuring apoE4 protein levels from dried blood spots (DBS) has the potential to provide information on genetic status as well as circulating levels and to include these measures in large survey settings.

METHODS: We developed a multiplex immunoassay to detect apoE4 protein levels in DBS from 15,974 participants, aged 50+ from Wave 6 of the Survey of Health, Ageing and Retirement in Europe (SHARE).

RESULTS: The apoE4 protein signal was presented in two separable distributions. One distribution corresponded to carriers of at least one copy of the ε4 allele. Fieldwork cofounders affected protein levels but did not explain individual differences.

DISCUSSION: Future research should investigate how genotype and apoE4 level interact with lifestyle and other variables to impact cognitive aging.

KEYWORDS
Alzheimer's disease, apolipoprotein E ε4, dried blood spots, Survey of Health, Ageing and Retirement in Europe, survey research

Nis Borbye-Lorenzen and Yacila I. Deza-Lougovski contributed equally to this study.

SHARE-COHESION: GA N°870628, SERISS: GA N°654221, SSHOC: GA N°823782; DG Employment, Social Affairs & Inclusion, Grant/Award Numbers: VS 2015/0195, VS 2016/0135, VS 2018/0285, VS 2019/0332, VS 2020/0313; German Ministry of Education and Research

1 | BACKGROUND

Considering the large societal and economic burden of Alzheimer's disease and related dementias (ADRD), the detection of biological markers indicating risk of cognitive decline is crucial for the identification of specific biological pathways that can be targeted by pharmacological interventions or changes in lifestyle. One such biological marker is the ε4 allele of apolipoprotein E (*APOE*), a well-documented genetic risk factor associated with dysregulation of brain lipids, decreased longevity, and increased prevalence of ADRD and cardiovascular diseases.[1–3] The detection of the *APOE* ε4 allele requires the extraction of a biological sample from the participant, thus its inclusion in clinical and epidemiological settings often faces challenges, and participants may show reluctance to consent to genetic analyses. A viable alternative is to identify the presence of circulating apoE4 protein in blood serum or plasma, without the need to perform a DNA analysis.[4–6] The information about the presence of the apoE4 protein (yes/no) can then be interpreted in line with studies using genetic information that distinguish carriers and non-carriers of the *APOE* ε4 allele. Along with genetic information about the *APOE* ε4 allele, circulating levels of the apoE4 protein might contain meaningful information regarding the metabolism of lipids and, consequently, cardiovascular morbidity and other risk factors for dementia.[7,8] However, because trained medical professionals need to perform the blood draw, this analysis is often not suitable for population-based settings.

In this study, we aim to overcome these limitations by developing a method to obtain apoE4 protein measurements in dried blood spot (DBS) samples. DBS is minimally invasive and can be implemented with little training by survey interviewers in the respondents' home.

We developed a multiplex immunoassay to detect apoE4 protein in DBS samples. Multiplex immunoassays are the method of choice when multiple protein biomarkers have to be measured in the same sample[9,10] with limited material. We combined the apoE4 assay with nine additional markers of interest for a broad assessment of the immune response in aging.[11]

To foreshadow the results, our assay provided a range of values depicted in two distributions, in line with the protein being highly detectable in carriers of the *APOE* ε4 allele and at the level of noise in non-carriers. To validate this interpretation further, we performed a targeted DNA analysis for the two single nucleotide polymorphisms (SNPs) defining the *APOE* genotype in a sub-sample of deceased SHARE participants to test our hypothesis that the two distributions corresponded to apoE4 positive and apoE4 negative and successfully generated a cut-off with excellent diagnostic accuracy.

2 | MATERIALS AND METHODS

2.1 | Sampling

This study used cross-sectional data of participants from SHARE, which is a pan-European, longitudinal, population-based study, which surveys economic, health-related, and social factors among people aged ≥ 50 with the aim to improve their quality of life with high-quality research.[12] The collection of DBS samples was implemented in Wave 6 in 2015. A total of 26,351 respondents from 12 countries (Israel, Spain, Greece, Italy, Switzerland, France, Slovenia, Belgium, Germany, Denmark, Estonia, Sweden) donated DBS samples. A detailed explanation of the sampling process is provided in a published SHARE working paper.[11]

For DBS collection, well-trained interviewers prepared blood collection during the SHARE interview. The puncture site on a participant's finger was pricked with a lancet. The first blood drop was discarded. The next blood drops were collected on barcode-labeled filter cards (Ahlstrom 226). DBS were left to dry until the end of the SHARE interview (≈ 25 minutes) before being packed with desiccant in plastic bags and mailed to the SHARE biobank (Odense, Denmark), where they were stored at −20°C until analyses.

Because the DBS samples were collected in non-clinical environments, various field conditions were recorded during DBS collection (i.e., drying time, shipment time, outside temperature, humidity protection). These field conditions were used for the initial quality control to ensure the integrity of the sample and later, to account for the influence of these variables on the apoE4 protein, as previous SHARE research showed that they might impact biomarker levels.[13,14] To do this, a multiple regression was carried out using log-transformed apoE4 levels as a dependent variable, whereas fieldwork conditions were used as predictors. Variables with a P value < 0.05 were considered significant. Statistical analyses were conducted using R software version 4.1.2 (https://www.R-project.org/).

2.2 | Consent statement and ethics

For DBS collection, respondents gave informed consent, which could be revoked at any time. Data collection was reviewed and approved by the ethics council of the Max Planck Society. Collection and treatment of blood samples was approved by country-specific ethics committees.

Regarding the genetic validation, SHARE does not perform genetic analyses from its respondents. Here, to validate the detection of the apoE4 protein in DBS, genotyping was carried out in DBS samples

of deceased participants from Denmark, Estonia, and Spain only and in accordance with Recital 27 of the General Data Protection Regulation (https://gdpr-info.eu/recitals/no-27), which clarifies that data protection regulations do not apply to data from deceased respondents. These countries allow scientifically necessary re-analyses. We also ensured that the selected respondents had consented to further analyses of their blood samples, including DNA.

2.3 | Sample extraction and protein analyses

Extracts from a large subset (N = 15,974) of the available DBS samples were obtained and assayed using a 10-plex immunoassay using preprinted Meso Scale plates (Meso Scale Diagnostics [MSD]) coated with antibodies specific for apoE4 and nine other biomarkers reported elsewhere.[11] As validation of the preprinted 10-plex, cross-reactivity was tested by adding one calibrator protein at a time together with all 10 detection antibodies to check for non-specific binding (NSB) in the nine remaining assays (NSB = [NSB signal – background signal]/[specific signal – background signal]). None of the 10 calibrators interfered significantly with the remaining nine assays (NSB < 5%).

The apoE4 assay was based on an apoE4-specific antibody for capture (#M067-3, MBL International) and an apoE-pan–specific antibody for detection (MSD) using recombinant Human ApoE4 (#JM-4699, MBL International) as calibrator. The preprinted MSD plates were incubated at room temperature (RT) with blocker A (#R93BA) for 30 minutes, then washed. Extracts were mixed 7:1 with 6× custom diluent (based on diluent 43, #R50AG, MSD) on the MSD plate. Calibrators were diluted in diluent 7 (#R54BB) and detection antibodies in diluent 3 (#R50AP, MSD). Controls were made in house from part of the calibrator solution in one batch, aliquoted in portions for each plate, and stored at −20°C until use. Extracts were incubated on the MSD plate for 2 hours at RT shaking at 0.6 g before washing and adding detection antibody after an additional 2 hours of incubation at RT and 0.6 g. The samples were read on the QuickPlex SQ 120 (MSD) 4 minutes after adding 2× Read buffer T (#R92TC, MSD). Analyte concentrations were calculated from the calibrator curves on each plate using 4PL logistic regression with the MSD Workbench software.

For analytical characterization, intra-assay variations (apoE4: 6.96%) were based on 24 replicate measurements of an internal control. Inter-assay variations (apoE4: 17.9%) were calculated from controls analyzed in duplicate on each plate during the sample analysis, 212 plates in total. Lower limits of detection were calculated as 2.5 standard deviations above the average of repeated measurements of the zero calibrator. The higher detection limit was defined as the highest calibrator concentration.

2.4 | Genetic validation

To validate the apoE4 protein measurements, *APOE* genotyping was performed on a smaller subset of DBS samples (N = 554). DNA was extracted using Exact-N-Amp lysis and neutralization kit (Sigma Aldrich). Samples were genotyped for the two *APOE* variants, rs7412 and rs429358, using commercial TaqMan assays (Thermo Fisher Scientific), assay ID C__904973_10 and C__3084793_20, respectively. The TaqMan analysis was performed on a Viia7 real-time polymerase chain reaction (PCR) instrument (Thermo Fisher Scientific) using the standard TaqMan PCR protocol. Five samples could not be analyzed due to instrument failure.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature for evidence of apolipoprotein E (apoE)4 detection in dried blood spots (DBS). Although there were several studies using this sampling method with other biomarkers, no study described specific analyses of apoE4 protein levels. Similarly, studies linking the *APOE* ε4 allele and peripheral apoE4 protein levels were mostly restricted to animals and small human samples. All the above-mentioned studies are cited in this report.
2. **Interpretation**: Our findings describe a novel method to detect apoE4 protein levels using limited sampling material, such as DBS. This will contribute to expanded use of biological markers to determine dementia risk or protective factors in large multinational and survey studies.
3. **Future directions**: Future studies can use the proposed method or similar methods to collect large biomarker information and to examine its interactions with lifestyle, social, and psychological aspects that may give rise to cognitive resilience or decline.

3 | RESULTS

In the 10-plex immunoassay, 13,108 samples fell within the assay limits of the apoE4 protein assay (3672–6,250,000 pg/mL; 2866 samples were below the detection limit) and appeared in two distributions (Figure 1). The assay is based on an apoE4-specific capture antibody and a detection antibody targeting apoE disregarding its isoform. We assumed that the high-value distribution reflected true-specific binding of the apoE4 protein, whereas the distribution at the lower end of values reflected non-specific cross-reactivity or binding to other *APOE* variants.

This assumption was validated on a subset of deceased participants (N = 549). The results of the genetic analysis guided us to a cut-off value of 30,000 pg/mL, dividing *APOE* ε4 carriers from non-carriers. Applying this cut-off resulted in 97% sensitivity and 98% specificity. The genetic analysis identified 2.37% homozygous (ε4/ε4 carriers) and 27.5% heterozygous (ε4/ε3 or ε4/ε2 carriers) for *APOE* ε4 (Figure 1, colored dots). When we apply this cut-off to the complete protein dataset, 24% of the participants with protein data were likely to carry at least one

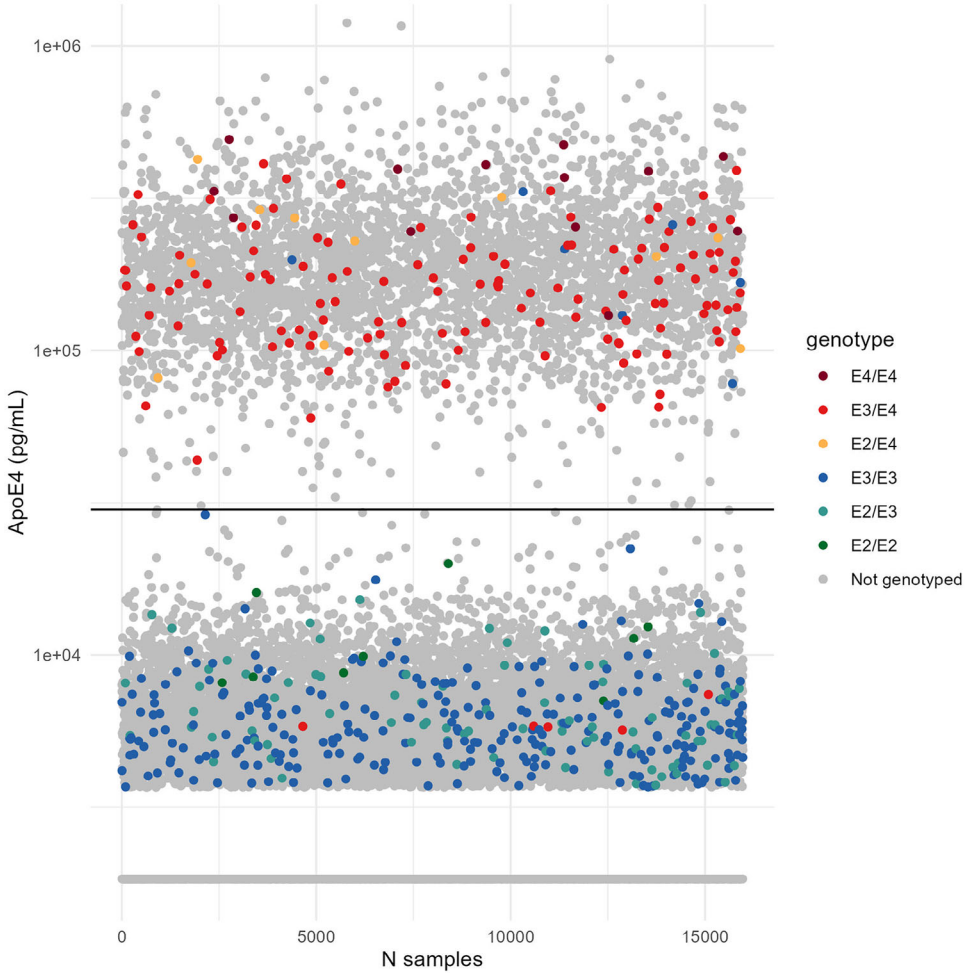

FIGURE 1 Raw data distributions of the apoE4 protein assay in SHARE DBS samples ($N = 15{,}974$) and genotyping of deceased SHARE respondents ($N = 549$) to define a cut-off. The *y* axis represents the levels of the apoE4 protein (in pg/mL) detected in the samples. Number of samples are depicted in the *x* axis. The anticipated apoE4 true specifics are in the upper part of the graph and the anticipated apoE4-negative samples are those in the bottom part of the graph. Each dot represents an apoE4 protein measurement with those genotyped shown in color and those not genotyped shown in gray. Carriers of one or two ε4 alleles (yellow, red, brown) were identified in the higher distribution, while carriers of other *APOE* alleles (ε2 and ε3) are found in the lower distribution of protein measurements (in green and blue). The line shows the cut-off at 30,000 pg/mL. *APOE*, apolipoprotein E; DBS, dried blood spots; SHARE, Survey of Health, Ageing and Retirement in Europe

copy of the *APOE* ε4 allele, in accordance with previous studies on the distribution of the *APOE* ε4 gene variant in European populations.[8,15,16]

Finally, a multiple regression to control for the effect of fieldwork conditions in the whole sample of *APOE* ε4 carriers ($N = 3837$) was performed. We observed that shipment time (in days: $\beta = -0.02$; 95% confidence interval [CI] = [−0.02, −0.01]; *P* value = < 0.001), outside temperature (in °C: $\beta = -0.01$; 95% CI = [−0.01, −0.00]; *P* value = < 0.001), and mean spot size (in cm^2: $\beta = 0.15$; 95% CI = [0.06, 0.24]; *P* value = < 0.001) significantly impact the levels of the circulating apoE4 protein (Table 1).

4 | DISCUSSION

Here, we used a multiplex immunoassay to quantify binding to the circulating apoE4 protein in dried blood spot samples of 50+ adults. Subsequently, we validated the results of the immunoassay and showed that 24% of the participants from the whole sample were likely to carry at least one copy of the *APOE* ε4 allele, which is highly consistent with large European studies.[16,17]

In humans, different methods to link the apoE4 isoform with the ε4 allele already exist,[4–6] but they have not been validated in DBS samples. The presented method thus enables the inclusion of a risk marker for ADRD, where DBS is the preferred sample material. Although the proposed method does not allow the distinction of *APOE* ε4 homozygous from heterozygous, it is already established that the presence of only one *APOE* ε4 allele is associated with an increased risk of dementia and cognitive decline.[2,16] This fact is crucial for large population-based studies examining health and cognition in aging societies.

In addition to providing a proxy for the *APOE* ε4 genetic status, quantitative apoE4 protein levels are also obtained. This finding is highly relevant for aging research, because studies of human plasma have

TABLE 1 Association between fieldwork conditions and apoE4 protein levels in SHARE.

$R^2 = 0.09$	Outcome: apoE 4 (log)		
Predictor (unit)	**Mean (SD), range**	**β coefficient**	**95% CI**
Shipment time (days)	4.96 (3.71), 1-30	−0.02	−0.02, −0.01*
Drying time (min)	22.8 (14.19), 1-83	0.00	−0.00, 0.00 ns
Outside temperature (°C)	13.90 (7.78), 0-35	−0.01	−0.01, −0.00*
Open/close bag (Y/N = 0/1)	0.09 (0.28)	−0.10	−0.21, 0.00 ns
Mean spot size (cm^2)	0.71 (0.41), 0.10-2.0	0.15	0.06, 0.24.*

Note: Observations were controlled for sex, age, and country, and weighted by the inverse of its probability of being sampled.
Abbreviations: apoE, apolipoprotein E; CI, confidence interval; ns, not significant; R^2, adjusted *R*-squared; SD, standard deviation; SHARE, Survey of Health, Ageing and Retirement in Europe.
*$P < 0.001$, $N = 3837$.

shown that, besides having a greater risk for dementia, *APOE* ε4 carriers have significantly lower levels of total plasma apoE attributable to a specific decrease in the apoE4 isoform.[8,15]

Finally, analysis of various field conditions in the present data showed that 9% of the variance of the apoE4 protein level is explained by these variables. It is not possible to avoid variability in fieldwork conditions like outside temperature or the shipment time to analysis centers from multiple countries. Our prior work shows that this is not specific to the apoE4 assay but also common for routine clinical markers.[13,14] Therefore, we recommend to always collect and control information regarding the environment and conditions for handling of the sample, and to include these potential confounders when working with DBS data.

In summary, our method constitutes a useful tool for investigating non-modifiable risk factors of cognitive aging. Applying it on blood samples collected in large population studies will support the development of preventive policies and early interventions, and thus strengthen the fight against the global burden of cognitive decline and dementia.

ACKNOWLEDGMENTS

We give a special thanks to Nicklas Petersen, Karin Skaarup, Karina Liebmann Madsen, Christof Gleie, and Malene Billsten Zent for their skillful performance in handling and analyzing the DBS samples. Yacila I. Deza-Lougovski and Anna Rieckmann are supported by an ERC Starting grant given to A.R. by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (ERC-STG-716065). This research was supported by NIA grants R01_AG063944 and R01_AG056329. Financial support for the genotyping came from the Max Planck Society. The SHARE data collection has been funded by the European Commission through FP5 (QLK6-CT-2001-00360), FP6 (SHARE-I3: RII-CT-2006-062193, COMPARE: CIT5-CT-2005-028857, SHARELIFE: CIT4-CT-2006-028812), FP7 (SHARE-PREP: GA N°211909, SHARE-LEAP: GA N°227822, SHARE M4: GA N°261982, DASISH: GA N°283646), and Horizon 2020 (SHARE-DEV3: GA N°676536, SHARE-COHESION: GA N°870628, SERISS: GA N°654221, SSHOC: GA N°823782) and by DG Employment, Social Affairs & Inclusion through VS 2015/0195, VS 2016/0135, VS 2018/0285, VS 2019/0332, and VS 2020/0313. Additional funding from the German Ministry of Education and Research, the Max Planck Society for the Advancement of Science, the U.S. National Institute on Aging (U01_AG09740-13S2, P01_AG005842, P01_AG08291, P30_AG12815, R21_AG025169, Y1-AG-4553-01, IAG_BSR06-11, OGHA_04-064, HHSN271201300071C, RAG052527A), and from various national funding sources is gratefully acknowledged (see www.share-project.org). We acknowledge financial support by Universität der Bundeswehr München. The funders mentioned above did not play any role in the study design, data analysis, decision to publish, or preparation of the manuscript.

Open access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest. Author disclosures are available in the supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Borbye-Lorenzen N, Deza-Lougovski Y, Holmgaard S, et al. Assessment of circulating apoE4 levels from dried blood spot samples in a large survey setting. *Alzheimer's Dement*. 2024;16:e12555. https://doi.org/10.1002/dad2.12555