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# CE(20:4) and CE(22:5) cholesteryl ester levels are elevated in the plasma of Alzheimer's disease patients with $\varepsilon 3/\varepsilon 4$ genotype

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Academic Editor: Ta-Yuan Chang, Geisel School of Medicine at Dartmouth College, USA

Received: December 15, 2024 Accepted: February 6, 2025 Published: February 24, 2025

**Cite this article:** Stefanini E, Serrano-Marín J, Iglesias A, Fernández MG, Sánchez-Navés J, Alkozi HA, et al. CE(20:4) and CE(22:5) cholesteryl ester levels are elevated in the plasma of Alzheimer's disease patients with  $\epsilon 3/\epsilon 4$  genotype. Explor Neuroprot Ther. 2025;5:100497. https://doi.org/10.37349/ent.2025.100497

## Abstract

**Aim:** This study investigates the association between *ApoE* genotype, plasma cholesteryl ester (CE) levels, and Alzheimer's disease (AD) status, with a focus on CE(20:4) and CE(22:5) levels as potential indicators of lipid metabolism alterations in AD patients.

**Methods:** Publicly available metabolomics data (DOI: 10.3233/JAD-215448) from 94 AD patients and 62 controls aged 55 and older were re-analyzed. CE levels were examined using non-parametric tests due to the non-normal overall data distribution. Comparisons were stratified by *ApoE* genotypes ( $\epsilon$ 3/ $\epsilon$ 3 and  $\epsilon$ 3/ $\epsilon$ 4).

**Results:** Significant increases in CE(20:4) and CE(22:5) levels were observed in AD patients with the *ApoE*  $\epsilon 3/\epsilon 4$  genotype compared to controls (*p*-values 0.0387 and 0.0348, respectively). No significant differences were found for other CEs or among  $\epsilon 3/\epsilon 3$  carriers (except for sex). Overlap between CE levels in AD and control groups limits their potential as diagnostic biomarkers but underscores their role in lipid dysregulation in AD pathophysiology.

**Conclusions:** Elevated CE(20:4) and CE(22:5) levels in  $\varepsilon 3/\varepsilon 4$  AD patients highlight lipid metabolism alterations associated with *ApoE* genotype. While not providing standalone biomarkers, these findings offer insights into AD-related lipid dysregulation and warrant further investigation in high-risk  $\varepsilon 4/\varepsilon 4$  carriers and therapeutic targeting of lipid metabolism.

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## **Keywords**

Metabolomics, biomarker, neurodegenerative diseases, cohort, cholesterol metabolism, cholesteryl esters, lipoproteins, lipidomics

## Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia, affecting millions of individuals worldwide. It is characterized by progressive cognitive decline, memory loss, and behavioral impairments, with hallmark pathological features including amyloid-beta (A $\beta$ ) plaques, tau neurofibrillary tangles, neuroinflammation, and synaptic dysfunction [1]. While the etiology of AD remains multifactorial, genetic predisposition plays a critical role in modulating risk and progression. Among the genetic factors, the apolipoprotein E (ApoE) gene is the most significant determinant of late-onset AD risk [2]. ApoE is a lipid transport protein involved in the regulation of cholesterol and lipid homeostasis, as well as the clearance of A $\beta$  from the brain. The three major ApoE isoforms— $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ —exert differential effects on these processes, with ApoE  $\epsilon 4$  associated with an increased risk of AD, while ApoE  $\epsilon 2$  provides protection [3]. Despite significant progress in understanding the role of ApoE in AD, its influence on systemic metabolic alterations remains underexplored.

Emerging evidence indicates that lipid metabolism is intricately linked to AD pathophysiology, particularly through its impact on membrane integrity, synaptic function, and amyloid deposition. Cholesteryl esters (CEs), the storage form of cholesterol in plasma, are increasingly recognized as key metabolites that may bridge the gap between ApoE function and AD pathology [4]. Transported within lipoproteins, CEs are influenced by the lipid-handling capacity of ApoE isoforms, suggesting that CE profiles may reflect both systemic and brain-specific lipid perturbations in AD [5]. Recent advances in metabolomics have enabled the comprehensive profiling of plasma metabolites, providing an opportunity to identify biomarkers and metabolic pathways associated with AD. In particular, lipidomics has highlighted changes in specific CE species that may inform disease mechanisms or predict risk in genetically predisposed individuals [6].

For this study, we took advantage of data obtained using novel metabolomics technologies that are available in the report by Kalecký et al., (2022) [7]. We re-analyzed data to investigate the relationship between *ApoE* genotype, plasma metabolite levels, and AD status. The dataset includes comparable data on the levels of multiple metabolites measured in the plasma from controls and AD patients, with the *ApoE* genotype recorded for everyone. While the dataset contains one case with an *ApoE*  $\epsilon^2/\epsilon^2$  genotype and few cases with the *ApoE*  $\epsilon^4/\epsilon^4$  (6 AD and 2 controls) genotype, it provides sufficient sample size to analyze metabolic differences in  $\epsilon^3/\epsilon^3$  and  $\epsilon^3/\epsilon^4$  combinations. Among the analyzed metabolites, we identified a significant increase in cholesteryl esters CE(20:4) and CE(22:5) in the plasma of AD patients with the *ApoE*  $\epsilon^3/\epsilon^4$  genotype compared to controls for other metabolites in either  $\epsilon^3/\epsilon^3$  or  $\epsilon^3/\epsilon^4$  combinations.

The identification of a novel association between two CEs and AD in *ApoE*  $\varepsilon 3/\varepsilon 4$  patients provides new insights into the interplay between lipid metabolism, *ApoE* genotype, and AD pathology. By leveraging metabolomics data from Kalecký et al., (2022) [7], we illustrate the potential of re-analyzing existing datasets to uncover genotype-specific metabolic signatures that can advance our understanding of AD mechanisms. Further studies are needed to confirm this finding and to investigate its relevance in high-risk individuals with the *ApoE*  $\varepsilon 4/\varepsilon 4$  genotype.

## **Materials and methods**

## **Data collection**

Data was retrieved from a previous study [7] in which a targeted metabolomics approach using the Biocrates MxP<sup>®</sup> Quant 500 system was undertaken using plasma and cerebral cortex samples from AD

patients and healthy controls. In this study, we re-analyzed only the data coming from plasma samples. The cohort is described as follows: "*Plasma samples were obtained from 94 AD cases and 64 control subjects, which were part of a longitudinal study conducted by the Texas Alzheimer's Research and Care Consortium (TARCC). The study was performed in 2005–2018 with participants, older than 55 years, recruited at dementia clinics of the TARCC member institutions. Controls included volunteers and family members" [7]. For the purposes of our analysis, the data was retrieved from the supplementary files; data from two controls (PX009 and PX025) were excluded due to missing metabolite levels, suggesting issues in the collection and/or processing of these samples. The set of data we have used and that is provided as Table S1, contains demographic data, <i>ApoE* genotype and comparable values of the level of the CEs that can be determined using Biocrates MxP<sup>®</sup> Quant 500. All individuals were older than 55 and part of a study conducted by TARCC (Texas Alzheimer's Research and Care Consortium) [8].

### **CE level data generation**

Using plasma from controls and patients, a targeted metabolomic approach was followed using the Biocrates MxP<sup>®</sup> Quant 500 targeted kit (Biocrates Life Sciences AG, Innsbruck, Austria). This kit can potentially quantify up to 524 lipids in flow-injection mode (FIA-MS/MS) and 106 small molecules in chromatography mode. Mass spectrometry is the procedure allowing the production of quantitative data. For this purpose, a triple quadrupole ultra-high-resolution liquid chromatograph and a tandem mass spectrometry coupled to a mass spectrometer were used. For our analysis, only data corresponding to the CEs detectable with the Biocrates kit were retrieved.

The study by Kalecký et al., (2022) [7] used Biocrates  $Met/DQ^{M}$  Oxygen-DB110-3005 for peak identification and computation of area under the peak. Further details of data production are given in the Kalecký et al., (2022) [7].

#### **Statistical analysis**

The analysis was performed in RStudio (2024.09.0+375), and included the usage of packages such as dplyr, tidyr, and ggplot2.

Taking the raw data in Table S1, normality tests were performed to select the methods for comparing data from patients and controls. For each CE mean was computed and comparisons were done using the Student's *t* test in case of normality of data; otherwise the Mann-Whitney test was used. It is important to note that for most CEs, the data did not follow a normal distribution.

Due to the low number of samples from  $\epsilon 2/\epsilon 2$  and  $\epsilon 4/\epsilon 4$  carriers, subsequent analysis focused on data filtered for  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes. Means and medians were calculated separately for each genotype and then for genotype and clinical condition. In this step, some CE metabolites such as CE(20:0), CE(22:2), and CE(22:6) showed a discrepancy between mean and median, suggesting asymmetric distributions or the presence of outliers. To confirm these observations, data distributions were assessed using the Shapiro-Wilk test for normality and the Levene test for homogeneity of variances. Only 5 out of 44 metabolite/genotype combinations met the criteria for normality and homogeneity of variance. For most data, non-parametric tests were required, confirming the non-normal nature of the distributions.

To evaluate the effect of sex on CE levels, we performed a separate analysis considering only subgroups where both males and females were present. Wilcoxon tests were applied to compare CE levels between sexes in patients and control subgroups with  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes. *p*-values were adjusted for multiple testing using the Benjamin-Hochberg method.

Fold changes [Alzheimer's disease/control (AD/NC)] were calculated for each metabolite in each genotype. The Kruskal-Wallis test was used to compare AD and NC for each metabolite within each genotype.

# Results

The data used for comparing CE data between samples of AD patients and control individuals and for statistical analysis considering *ApoE* genotype is provided in Table S1. Table S1 contains data retrieved from the supplementary files of the Kalecký et al., (2022) [7]. With few exceptions, the values for both AD and control groups do not follow a Gaussian distribution. The list with means for all the CEs is in Table 1, which also shows the fold change of the mean of AD versus controls. While the mean levels of many CEs are similar between AD and control groups, the most pronounced differences were observed for CE(16:1), CE(20:0), CE(20:1), CE(22:2) and CE(22:5). Setting the threshold in 0.8 and 1.2, the compounds with lower fold change (AD versus controls) were CE(20:0), CE(20:1) and CE(22:2), and those with greater fold change were CE(16:1) and CE(22:5) (Table 1). Table 2 provides the statistical parameters for the CEs that have more distant mean comparing patients and controls, regardless of *ApoE* genotype. By ANOVA in case of normal distribution or by the Kruskal-Wallis test in the absence of normality the *p*-values for all comparisons were > 0.05. This indicates that no CE could be identified to distinguish between patients and healthy controls based on mean difference alone.

CE	Mean_AD	Mean_NC	Fold_Change	
CE(14:0)	0.0257	0.0246	1.0460	
CE(14:1)	0.0013	0.0012	1.0662	
CE(15:0)	0.0060	0.0061	0.9747	
CE(15:1)	0.0006	0.0005	1.1022	
CE(16:0)	0.1442	0.1451	0.9939	
CE(16:1)	0.0821	0.0697	1.1787	
CE(17:0)	0.0046	0.0046	0.9983	
CE(17:1)	0.0048	0.0046	1.0605	
CE(18:0)	0.0221	0.0219	1.0072	
CE(18:1)	0.2928	0.2839	1.0311	
CE(18:2)	1.7756	1.7135	1.0363	
CE(18:3)	0.0944	0.0865	1.0910	
CE(20:0)	0.0048	0.0072	0.6701	
CE(20:1)	0.0024	0.0037	0.6431	
CE(20:3)	0.0355	0.0339	1.0502	
CE(20:4)	0.4732	0.4286	1.1039	
CE(20:5)	0.0717	0.0736	0.9737	
CE(22:0)	0.0004	0.0005	0.8597	
CE(22:1)	0.0004	0.0004	0.8790	
CE(22:2)	0.0011	0.0015	0.7194	
CE(22:5)	0.0047	0.0041	1.1495	
CE(22:6)	0.0414	0.0428	0.9677	

Table 1. Comparative mean values for CEs and fold change [Alzheimer's disease (AD) versus control (NC)]

Table 2. Mean, standard deviation (SD), and statistical parameters in Alzheimer's disease (AD	)) and control cases for
CE(16:1), CE(20:0), CE(20:1), CE(22:2), and CE(22:5)	

CE	AD (SD)	Control (SD)	T-statistic	<i>p</i> -value
CE(16:1)	0.082 (0.040)	0.067 (0.042)	0.784	0.436
CE(20:0)	0.0050 (0.0038)	0.0069 (0.026)	-0.961	0.343
CE(20:1)	0.0024 (0.0018)	0.0036 (0.013)	-1.011	0.319
CE(22:2)	0.0011 (0.00078)	0.0014 (0.0060)	-0.840	0.406
CE(22:5)	0.0047 (0.0016)	0.0039 (0.0020)	1.040	0.302

Subsequently, a similar approach was undertaken but stratifying by *ApoE* genotype. Since in the cohort there were only one individual with *ApoE*  $\epsilon 2/\epsilon 2$  genotype and two controls with *ApoE*  $\epsilon 4/\epsilon 4$  genotype, we focused solely on data from those individuals having  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes. The number of samples of AD patients with  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes was, respectively, 50 and 37. The number of control samples with  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes was 36 and 24, respectively. The median value for each CE was computed for all individuals and stratified by *ApoE* genotype. Due to the large variability of data, pairwise comparisons were performed using the Kruskal-Wallis test. Table 3 shows the median stratified by genotype.

Metabolite	AD (ε3/ε3)	AD (ε3/ε4)	ΝC (ε3/ε3)	ΝC (ε3/ε4)
CE(14:0)	0.023643	0.024007	0.023225	0.017911
CE(14:1)	0.001242	0.001078	0.001151	0.000897
CE(15:0)	0.005566	0.006095	0.005907	0.005832
CE(15:1)	0.000476	0.000526	0.000471	0.000480
CE(16:0)	0.122656	0.128423	0.130894	0.134049
CE(16:1)	0.072250	0.062645	0.064884	0.049666
CE(17:0)	0.004572	0.004669	0.004495	0.004566
CE(17:1)	0.004720	0.004743	0.004483	0.003734
CE(18:0)	0.020752	0.021065	0.021088	0.020039
CE(18:1)	0.277685	0.274327	0.278495	0.247032
CE(18:2)	1.788830	1.763925	1.749266	1.566733
CE(18:3)	0.084403	0.080391	0.082900	0.069042
CE(20:0)	0.003211	0.003029	0.002426	0.002688
CE(20:1)	0.001617	0.001664	0.001423	0.001393
CE(20:3)	0.034856	0.033943	0.034998	0.027941
CE(20:4)	0.432905	0.516135	0.378761	0.412623
CE(20:5)	0.052278	0.046796	0.057388	0.064487
CE(22:0)	0.000407	0.000386	0.000357	0.000324
CE(22:1)	0.000305	0.000280	0.000290	0.000341
CE(22:2)	0.000652	0.000651	0.000443	0.000450
CE(22:5)	0.004363	0.004429	0.003872	0.003546
CE(22:6)	0.036965	0.034429	0.040133	0.043817

Table 3. Median depending on disease [Alzheimer's disease (AD) or healthy control (NC)] and genotype

No significant differences in CE levels were detected between AD patients and controls carrying the  $\epsilon 3/\epsilon 3$  genotype. The statistical analysis considered some less relevant comparisons, such as comparing AD with  $\epsilon 3/\epsilon 3$  versus controls with  $\epsilon 3/\epsilon 4$  (or vice versa), which were not informative. In contrast, when the  $\epsilon 3/\epsilon 4$  was considered, significantly different medians were found for CE(20:4) and CE(22:5) with *p*-values of 0.0387 and 0.0348, respectively.

Box plots of the levels of these two CEs for patients and controls with the  $\epsilon 3/\epsilon 4$  genotype (Figure 1) show marked overlap meaning that none of these two CEs can be considered as a biomarker of AD. While the levels of these two CEs tend to be higher in AD patients with  $\epsilon 3/\epsilon 4$  genotype compared to controls, no threshold could achieve enough sensitivity and specificity to make them useful diagnostic markers. In the box plots in Figure 1 it is noticeable the difference in median between patients (red) and controls (blue) in the case of CE(20:4) and of CE(22:5). In contrast, the median value of the box plot for CE(18:0) is very similar in AD and controls. The median values for the remaining CEs were similar when comparing patients and controls with the same genotype. In summary, although no CE can be considered a biomarker for AD, the median levels of CE(20:4) and CE(22:5) were significantly different between AD cases and controls with the  $\epsilon 3/\epsilon 4$  genotype.



Figure 1. Box and whiskers plots of the plasma levels of CE(18:0), CE(20:4), and CE(22:5) in controls and in patients. The horizontal line in each box is the media, which indicates the middle quantile (50th percentile). Top and bottom extremes of the boxes indicate, respectively, the 75% and 25% quartiles. The points extending outside the whiskers are outliers

The analysis of sex differences in CE levels revealed that most CEs did not show significant variation between males and females. However, CE(22:5) exhibited significant sex differences in both controls and patients with the *ApoE*  $\epsilon 3/\epsilon 3$  genotype (*p*-values of 0.0370 and 0.0239, respectively), while CE(20:4) showed no significant differences.

## **Discussion**

In this study, we re-analyzed publicly available metabolomics data from [7], which includes plasma metabolite levels from controls and AD patients with known *ApoE* genotypes. This dataset provides a robust sample size for analyzing metabolic changes in individuals carrying the *ApoE*  $\epsilon 3/\epsilon 3$  and  $\epsilon 3/\epsilon 4$  genotypes. Among the metabolites analyzed, we identified a significant increase in the median of CE(20:4) and CE(22:5) in AD patients with the *ApoE*  $\epsilon 3/\epsilon 4$  genotype compared to controls with the same genotype. Importantly, no significant differences were observed for other metabolites or genotype combinations. However, the substantial overlap in CE levels between patients and controls limits their potential as diagnostic markers. Instead, their tendency to be elevated in patients reflects lipid metabolic alterations associated with the disease, particularly in the context of  $\epsilon 3/\epsilon 4$  carriers.

There is evidence that CE levels vary in patients with neurological disturbances. For example, the overall CE levels are elevated in the caudate and putamen nuclei of patients with Huntington's disease [9]. Given the wide structural diversity of CEs, which is determined by the size and number of double bonds of their acyl side chains, it remains a challenge to identify which specific compounds are increased or decreased in disease states. Some CEs may emerge as potential biomarkers when analyzed in plasma or cerebrospinal fluid. A small randomized trial of supplementation with docosahexaenoic acid, a polyunsaturated fatty acid, showed that certain CE levels in plasma and/or cerebrospinal fluid increased upon supplementation, with *ApoE* genotype influencing the dynamics of these changes [10]. Interestingly, when combining data from five studies on healthy aging, the plasma level of the two CEs detected here,

CE(20:4) and CE(22:5), was significantly different in individuals with *ApoE*  $\epsilon 2/\epsilon 2$  genotype compared with those with  $\epsilon 3/\epsilon 3$  genotype. There were no reliable results concerning the *ApoE*  $\epsilon 4/\epsilon 4$  genotype due to the study's design, which prioritized maximizing the number of  $\epsilon 2$  carriers while minimizing the number of  $\epsilon 4$  carriers [11].

Our results also suggest that CE(22:5) may be influenced by sex in individuals with the *ApoE*  $\varepsilon 3/\varepsilon 3$  genotype, indicating a potential interaction between lipid metabolism and biological differences. However, as this effect was not observed in all subgroups, further analyses in larger cohorts are needed to clarify its significance or lack thereof.

The small number of individuals with the *ApoE*  $\epsilon 2/\epsilon 2$  and *ApoE*  $\epsilon 4/\epsilon 4$  genotypes, restricted our ability to perform detailed subgroup analyses. Additionally, the lack of absolute metabolite concentrations prevents direct comparisons of CE levels in absolute terms. However, the values in Table S1 remain comparable, as they were derived using mass spectrometry with an area-under-the-curve approach and appropriately normalized. Therefore, our conclusions regarding differences—or the absence thereof—in CE levels based on disease status and *ApoE* genotype remain robust.

This study highlights the value of re-analyzing existing metabolomics datasets to uncover novel insights, particularly when genotype-specific associations may be masked in mixed-population analyses. Although we were unable to directly analyze  $\varepsilon 4/\varepsilon 4$  patients due to the limited sample size (n = 4), it is reasonable to predict that CE(20:4) and CE(22:5) concentrations would also be elevated in this high-risk group. This expectation aligns with the well-documented inefficiency of *ApoE*  $\varepsilon 4$  in lipid clearance and cholesterol homeostasis [12]. The potential elevation of CE(20:4) and CE(22:5) in  $\varepsilon 4/\varepsilon 4$  individuals highlights the importance of targeted studies in this high-risk group to validate these predictions and explore therapeutic interventions aimed at modulating lipid metabolism. Combining CE(20:4) and CE(22:5) plasma concentrations with other metabolic or clinical parameters could enhance our understanding of AD-related lipid dysregulation [13]. Larger cohort studies, including those with  $\varepsilon 4/\varepsilon 4$  individuals, are necessary to validate these results and explore their broader implications.

The observed increase in the median level of two CEs aligns with the hypothesis that systemic lipid dysregulation contributes to AD pathophysiology [14]. CEs play crucial roles in lipid transport and storage, and their profiles are shaped by the lipid-handling efficiency of ApoE isoforms [15]. The *ApoE*  $\epsilon$ 4 allele, associated with impaired lipid metabolism and increased AD risk [16], likely drives the increased CE(20:4) and CE(22:5) levels in  $\epsilon$ 3/ $\epsilon$ 4 individuals. These findings underscore the need for genotype-stratified analyses to reveal metabolic changes that may be obscured in mixed-genotype populations [17]. It should be noted that the two CEs detected here have in common an acyl with a large number of carbon atoms, 20 and 22, and that the acyl is highly unsaturated, 4 and 5 double bonds for, respectively, CE(20:4) and CE(22:5).

Despite these insights, this study has several limitations that must be acknowledged. First, we relied on publicly available data, which, while valuable, lacks an independent validation cohort. The absence of a secondary dataset limits the generalizability of our findings, underscoring the need for replication in larger, well-characterized population cohorts. Furthermore, while plasma metabolomics provides a systemic view of metabolic alterations, it does not directly reflect changes occurring in the brain. Given the critical role of lipid metabolism in neurodegenerative diseases, further studies should examine CE levels in cerebrospinal fluid or brain tissue to determine whether similar alterations are present at the central level. Additionally, the lack of detailed neuropathological data, such as brain tissue analysis or Braak staging, limited our ability to fully investigate the relationship between CE levels and disease progression.

In conclusion, the elevated median levels of CE(20:4) and CE(22:5) in  $\varepsilon 3/\varepsilon 4$  AD patients highlight the role of lipid dysregulation in the disease but do not serve as reliable biomarkers for distinguishing AD patients from controls. The clinical significance of this finding lies in its potential to drive further investigations into the role of CEs in AD. A deeper understanding of these metabolic alterations could provide new insights into disease mechanisms and inform strategies for modulating lipid metabolism in genetically predisposed populations.

# Abbreviations

AD: Alzheimer's disease Aβ: amyloid-beta CEs: cholesteryl esters

# **Supplementary materials**

The supplementary table for this article is available at: https://www.explorationpub.com/uploads/Article/file/100497\_sup\_1.xlsx.

## **Declarations**

## Acknowledgments

Special thanks to all the participants that included professors and students of the University of Barcelona and of the EÒLIA Centre Superior d'Art Dramàtic. We would like to thank the CCiTUB (Scientific and Technological Centers of the UB) of the Universitat de Barcelona (Barcelona, Spain), for the expert and technical help using the LC-MS/MS equipment.

## **Author contributions**

ES: Data curation, Formal analysis, Investigation, Visualization, Writing—original draft. JSM: Data curation, Formal analysis, Investigation, Writing—original draft. AI: Validation, Writing—original draft. MGF: Validation, Methodology, Visualization, Writing—original draft. JSN and HAA: Conceptualization, Writing original draft. DBC: Validation, Conceptualization, Supervision, Writing—original draft. RF: Conceptualization, Supervision, Writing—original draft.

## **Conflicts of interest**

Rafael Franco, who is the Editor-in-Chief of Exploration of Neuroprotective Therapy, was not involved in the decision-making or the review process of this manuscript. The other authors declare no conflict of interest.

### **Ethical approval**

This study did not involve new human or animal experiments. According to the regulations of the institution and regional government guidelines, the use de-identified data, which are already reported and readily available, does not require specific approval by an Ethics Committee.

### **Consent to participate**

Not applicable.

**Consent to publication** 

Not applicable.

### Availability of data and materials

The data used in this study from the Kalecký et al. (2022) study, under a Creative Commons Attribution Non-Commercial (CC BY-NC 4.0) License, is provided in supplementary Table S1. The remaining supporting data of this study are available from the corresponding author upon reasonable request.

### Funding

Not applicable.

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