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Serum TCA cycle metabolites in Lewy bodies dementia and Alzheimer's disease: Network analysis and cognitive prognosis

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ABSTRACT

Abnormalities in the Tri-Carboxylic-Acid (TCA) cycle have been documented in dementia. Through network analysis, TCA cycle metabolites could indirectly reflect known dementia-related abnormalities in biochemical pathways, and key metabolites might be associated with prognosis. This study analyzed TCA cycle metabolites as predictors of cognitive decline in a mild dementia cohort and explored potential interactions with the diagnosis of Lewy Body Dementia (LBD) or Alzheimer's Disease (AD) and APOE-ε4 genotype. We included 145 mild dementia patients (LBD = 59; AD = 86). Serum TCA cycle metabolites were analyzed at baseline, and partial correlation networks were conducted. Cognitive performance was measured annually over 5-years with the Mini-mental State Examination. Longitudinal mixed-effects Tobit models evaluated each baseline metabolite as a predictor of 5-years cognitive decline. APOE-ε4 and diagnosis interactions were explored. Results showed comparable metabolite concentrations in LBD and AD. Multiple testing corrected networks showed larger coefficients for a negative correlation between pyruvate – succinate and positive correlations between fumarate – malate and citrate – Isocitrate in both LBD and AD. In the total sample, adjusted mixed models showed significant associations between baseline citrate concentration and longitudinal MMSE scores. In APOE-ε4 carriers, baseline isocitrate predicted MMSE scores. We conclude that, in mild dementia, serum citrate concentrations could be associated with subsequent cognitive decline, as well as isocitrate concentrations in APOE-ε4 carriers. Down-regulation of enzymatic activity in the first half of the TCA cycle (decarboxylating dehydrogenases), with upregulation in the latter half (dehydrogenases only), might be indirectly reflected in serum TCA cycle metabolites' networks.

1. Introduction

Brain energy and metabolism have been closely related to mitochondrial function, and several mitochondrial abnormalities in the tricarboxylic acid (TCA) cycle and electron transport complex (ETC) are well-recognized mechanisms linked to neurodegeneration (Mahley and

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Huang, 2012). *In vitro* and *in vivo* studies point to a link between dysfunction in mitochondrial energy metabolism and amyloidogenesis in both Alzheimer's Disease (AD) (Atamna and Frey, 2007) and Lewy body dementia (LBD) (Spano et al., 2015). Furthermore, mitochondrial function is linked to neuronal survival and autophagy in AD (Kshirsagar et al., 2021; Moreira et al., 2010).

Human studies assessing the role of the TCA cycle in neurodegeneration have been mainly conducted in AD postmortem brain tissues, measuring enzyme activity and expression. These findings supported deficient enzyme activity in the pyruvate, Isocitrate, and α-ketoglutarate dehydrogenase complexes, as well as the cytochrome oxidase (COX, so-called Complex IV). On the other hand, increased activity in succinate dehydrogenase (so-called Complex II) and malate dehydrogenase was observed (Blass et al., 2000; Bubber et al., 2005). Although mitochondrial dysfunction is implicated in all forms of neurodegeneration, few studies have evaluated TCA cycle metabolites in blood as potential biomarkers of clinical prognosis in patients with mild dementia.

Most studies have quantified either TCA cycle metabolites concentration or enzyme activity *in vivo,* mainly from plasma and platelets, focused on AD and aging effects (de Leeuw et al., 2021; Cheng et al., 2015; Lawton et al., 2008; Wilkins et al., 2017). In serum samples, evidence is not as extensive, and less is known in the early stages of AD and LBD. As a case in point, a small metabolomic study used capillary electrophoresis-mass spectrometry based on serum and saliva samples from 10 patients with various neurodegenerative dementias. Among the quantified metabolites, the investigators found increased serum isocitrate concentrations in patients with dementia when compared to age-matched healthy controls (Tsuruoka et al., 2013). Still, mitochondrial dysfunction may behave differently according to the predominant brain pathology or different genotypes. Thus, the Apolipoprotein epsilon 4 allele of the APOE gene (APOE-ε4) has been recently associated with significant alterations in brain energy metabolism (Yan et al., 2020), and isocitrate serum concentration has been associated with an increased risk of Mild Cognitive Impairment (MCI) in APOE-ε4 carriers (He et al., 2022). TCA cycle metabolite analyses in clinical studies with a robust diagnostic evaluation are needed to conduct prognostic studies on different types of dementia. Also, proper stratification according to the presence of the APOE-ε4 alleles would be highly informative in evaluating TCA cycle metabolites as predictors of disease progression in patients with dementia.

Metabolomic profiling is frequently analyzed via correlation-based networks that facilitate the inference of potential biological relationships in the data (Iqbal et al., 2018), elucidating the potential organization of metabolically functional modules (i.e., highly connected metabolites) that might reflect known biochemical pathways (Fukushima et al., 2011). In addition to the potential prognostic value of TCA cycle metabolites, network modeling can contribute to elucidating the interdependencies between TCA cycle metabolites, facilitating mechanistic interpretations and providing potential indirect measurements of altered biochemical pathways (Perez De Souza et al., 2020; Johnson et al., 2016).

With all the above, this study aims first to analyze whether TCA cycle metabolites concentration in mild dementia predicts cognitive decline. Secondly, we aim to explore whether TCA cycle metabolites predict cognitive decline differently in patients with AD or LBD and patients with or without APOEε4. Finally, we aim to describe the metabolite-metabolite correlations using network analysis according to diagnosis and APOE-ε4.

2. Materials and methods

2.1. Study design and setting

This is a secondary analysis of the "The Dementia Study of Western Norway" (DemVest) cohort. A detailed description of the design, recruitment, and assessments in the primary study is stated elsewhere (Aarsland et al., 2008). Briefly, the DemVest cohort recruited mild dementia patients due to different etiologies from 2005 to 2007 from all dementia clinics in Hordaland and Rogaland counties in Norway. All participants underwent annual follow-up until death, with very low attrition for other reasons (Aarsland et al., 2008). The regional ethics committee and Norwegian health authorities approved the DemVest study. All patients signed informed consent prior to inclusion.

2.2. Participants

Exclusion criteria (examined at baseline) in the DemVest study consisted of absence of dementia, moderate or severe dementia, delirium, history of bipolar or psychotic disorder, terminal illness, or recently diagnosed major somatic disease.

For the current analysis, 145 patients with a final diagnosis of dementia due to LBD or AD (as outlined below, Section 2.3) with available serum TCA cycle metabolites quantification were included. Clinical data from the first five years (i.e., Baseline + 5 annual follow-ups) was analyzed.

2.3. Clinical assessment

Initial diagnosis of dementia was made according to DSM-IV criteria (Diagnostic and statistical manual of mental disorders, 1994), and subsequent dementia subtyping diagnosis was made through consensus based on operationalized diagnostic criteria for AD and LBD. Subjects were classified as AD (according to the National Institute of Neurological and Communicative Disorders, Stroke-Alzheimer's Disease and Related Disorders Association/NINCDS-ADRDA) (McKhann et al., 1984), Dementia with Lewy Bodies (DLB) (McKeith et al., 2005), or Parkinson's Disease Dementia (PDD) (Emre et al., 2007) (according to specific International consensus criteria). Four clinical specialists independently applied diagnostic criteria, revising the diagnosis of the patients at each visit (i.e., yearly). The clinical specialists' group evaluated changes in the diagnosis during the follow-up, and the final diagnosis was determined through consensus giving priority to neuropathological confirmation when available. High consistency between the clinical and neuropathological diagnosis has been previously reported in the DemVest cohort (Aarsland et al., 2008; Skogseth et al., 2017).

Considering the overlapping in genetics, neuropathology, various clinical features, and management between PDD and DLB, these two subsamples (PDD = 8; DLB = 51) were combined into a single LBD group (Ka et al., 2018; Armstrong, 2019).

At baseline, patients with a Mini-mental State Examination (MMSE) (Folstein et al., 1975) total score greater or equal to 20 or with a Clinical Dementia Rating scale (CDR) (Hughes et al., 1982) global score equal to one were considered at the mild stage of dementia.

Global cognitive performance (as an indicator of cognitive decline) was evaluated with the MMSE total score across study time points.

2.4. TCA cycle metabolites quantification

Pyruvate and six TCA cycle intermediates (i.e., citrate, Isocitrate, α-ketoglutarate, succinate, fumarate, malate) were quantified from serum samples using Waters Acquity UPLC - TQ-XS mass spectrometer instrumentation. Proteins and phospholipids were removed from the serum samples with a Waters Ostro™ Pass-through sample preparation plate according to the supplier's manual. The purified plasma samples were dried with Speedvac (4 Torr, 60° C 2,5h) and kept at −25 °C until further analysis. Complete information on the derivatization step and LC-MS/MS method can be found elsewhere (Røst et al., 2020). There were no thaw-freeze cycles prior to analysis.

Absolute TCA cycle metabolites concentrations were log-transformed, standardized, and centered to the mean, in order to achieve normal distributions for subsequent analyses.

2.5. Statistical analysis

Baseline diagnosis-related differences in demographics (i.e., age, gender, and years of education) were analyzed using independent samples t-tests for continuous variables and Chi-square tests for categorical variables. Baseline differences in MMSE and CDR (sum of boxes) were evaluated using the Mann-Whitney *U* test.

Baseline diagnosis-related differences in the concentration of TCA cycle intermediates were estimated using independent samples unequal variance t-tests, followed by multiple testing correction using the False Discovery Ratio (FDR) (Benjamini and Hochberg, 1995). Partial-Least Squares Discriminant Analysis (PLS-DA) using MetaboAnalyst 5.0 web tool (Pang et al., 2021) was also conducted to test for group-related differences in metabolites concentration.

Metabolite-metabolite network analysis was conducted in the total sample using partial correlations networks implemented in qgraph and bootnet (Epskamp et al., 2012; Epskamp et al., 2018). Thus, each of the quantified TCA cycle metabolites was represented as a node within a network, and the pairwise node dependencies were plotted as edges linking them. The effect size for these dependencies was reported as partial correlation coefficients. Non-regularized partial correlation networks (i.e., non-regularized Gaussian Markov random field) for TCA cycle metabolites in the total sample were computed for the main analysis (Epskamp et al., 2018). The resulting p-values from the above correlation coefficients were also corrected for multiple testing with the FDR method, and only edges with q-values < 0.05 were plotted in the results (Benjamini and Hochberg, 1995). Additional subgroup network analyses were conducted by diagnosis (LBD and AD), APOE-ε4 genotype (Any ε4 allele vs. No ε4 allele carrier). As large group differences only can be observed in network models with a sample size equal to or >100–200 subjects per group (Haslbeck, 2022) and considering the descriptive nature of this step, network models were not compared by diagnosis or APOE-ε4 subgroups.

Finally, in the total sample of mild dementia, we analyzed the association between the concentration of each TCA cycle metabolite (measured at baseline) and cognitive decline (measured at baseline + 5 annual follow-ups). Thus, longitudinal mixed-effects Tobit models were performed with longitudinal MMSE scores as the outcome variable. In separate models, each baseline metabolite was analyzed as the predictor of cognitive decline (i.e., longitudinal MMSE scores). The models were adjusted by time, age, gender, APOE-ε4, and diagnosis (as well as significant time interactions). Metabolite*time interactions with APOE-ε4 and diagnosis were also explored in independent models. The Huber-White sandwich estimator was used to obtain robust standard errors. In these mixed models, missing data during follow-up was addressed under the missing-at-random assumption (Ibrahim and Molenberghs, 2009). The above models were corrected for multiple testing, and FDR (q-values) were reported.

Network analyses were conducted using RStudio © version 1.1.456 with qgraph, fdrtool, and bootnet packages (Epskamp et al., 2012; Epskamp et al., 2018; RStudio Team, 2019; Strimmer, 2008). Mixed-effects models were implemented in Stata Statistical Software: Release 15. StataCorp. 2017. College Station, TX: StataCorp LLC.

The current study is reported following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (Vandenbroucke et al., 2007).

3. Results

In the current analysis, the cohort of mild dementia patients included 145 subjects (LBD = 59; AD = 86). The number of participants at each follow-up stage (and the dropout rate) is depicted in Supplementary Fig. 1. In the mild dementia cohort, the mean follow-up time (in years) at each time point was as follows: Baseline (Mean = 0 SD = 0.004), Year 1 (Mean = 1.10 SD = 0.19), Year 2 (Mean = 2.16 SD = 0.22), Year 3 (Mean = 3.22 SD = 0.23), Year 4 (Mean = 4.26 SD = 0.27), Year 5 (Mean = 5.30 SD = 0.31), see Supplementary Fig. 2.

There were no differences in baseline demographic characteristics such as age and education when comparing LBD and AD subgroups, except for significant differences in gender proportion. There were no significant differences between LBD and AD subgroups for MMSE, CDR – Sum of boxes scores, or APOE-ε4 allele frequencies (Table 1).

3.1. Tca cycle metabolites analysis

At baseline, all TCA cycle metabolite concentrations were comparable between LBD and AD subgroups with non-significant results for both t-tests (Fig. 1) and PLS-DA (Supplementary Fig. 3 and Supplementary Table 1). TCA cycle metabolites were comparable in men and women, as well as APOE- ε4 subgroups ($p > 0.05$, results not shown).

Network models for the total mild dementia cohort are presented in Fig. 2A. Each TCA cycle metabolite was represented as a node. Only the significant edges (after multiple testing corrections) were plotted (i.e., edges with q-values < 0.05). Non-regularized network models exhibited moderate to high correlations between Pyruvate – Succinate (edge weight = −0.40), Fumarate – Malate (edge weight = 0.74), and Citrate – Isocitrate (edge weight = 0.47).

In line with the above interdependencies, both LBD and AD subgroups showed similar significant metabolite correlations for Pyruvate – Succinate, Fumarate – Malate, and Citrate – Isocitrate. The independence of α-ketoglutarate was observed only in the AD network. The

Table 1
Baseline and Follow-up characteristics of the total sample and diagnosis subgroups.

Baseline	Total sample (N = 145)	AD (N = 86)	LBD (N = 59)	p-value
Age (S.D)	75.32 (7.09)	75.33. (7.76)	75.31 (6.06)	0.988
Gender				0.007
Women (%)	81 (55.86)	56 (65.12)	25 (42.37)	–
Men (%)	64 (44.14)	30 (34.88)	34 (57.63)	–
Any APOE4 allele (%)	80 (55.17)	47 (54.65)	33 (55.93)	0.615
Years of education (S.D)	9.70 (2.97)	9.76 (3.15)	9.61 (2.68)	0.763
MMSE (IQR)[a]	23 (22–26)	23 (22–25)	24 (21–26)	0.517
CDR-SB (IQR)[a]	10 (6–14)	9 (6–14)	11 (6–13)	0.986
Follow-up	Total sample (N = 145)	AD (N = 86)	LBD (N = 59)	p-value a
MMSE (IQR)				
Year 1	23 (22–26)	23 (22–25)	24 (21–26)	0.466
Year 2	22 (18.8–24)	22 (19–24)	21.5 (16.5–25)	0.088
Year 3	19 (4–22.5)	19 (16–22.8)	17 (12–22)	0.597
Year 4	16 12.2 21	16 (13–21)	17 (8.5–20.5)	0.356
Year 5	13 7 19.8	13 (6.3–19.8)	15 8.5 19.2	0.635
CDR-SB (IQR)				
Year 1	8 5 11	8 5 11.2	7.5 4.5 10.5	0.621
Year 2	9 6 13	9 6.5 13.2	8 5.5 11.9	0.100
Year 3	9 5 13	8 5 12	10.5 6 15	0.022
Year 4	8.8 5.1 12.8	8 5 13	9 6 12	0.964
Year 5	8 4.5 14	8 4.5 14	8.5 5 13	0.832

AD: Alzheimer's Disease. LBD: Lewy body Dementia. S.D: Standard Deviation. IQR: Interquartile range. MMSE: Mini-Mental State Examination. CDR-SB: Clinical Dementia Rating – Sum of Boxes. APOE4: Apolipoprotein E4.
Values are presented as mean (S.D) and frequency (%) for gender.
P - values < 0.05 are printed in bold.
[a] Values are presented as median (IQR), Mann-Whitney U.

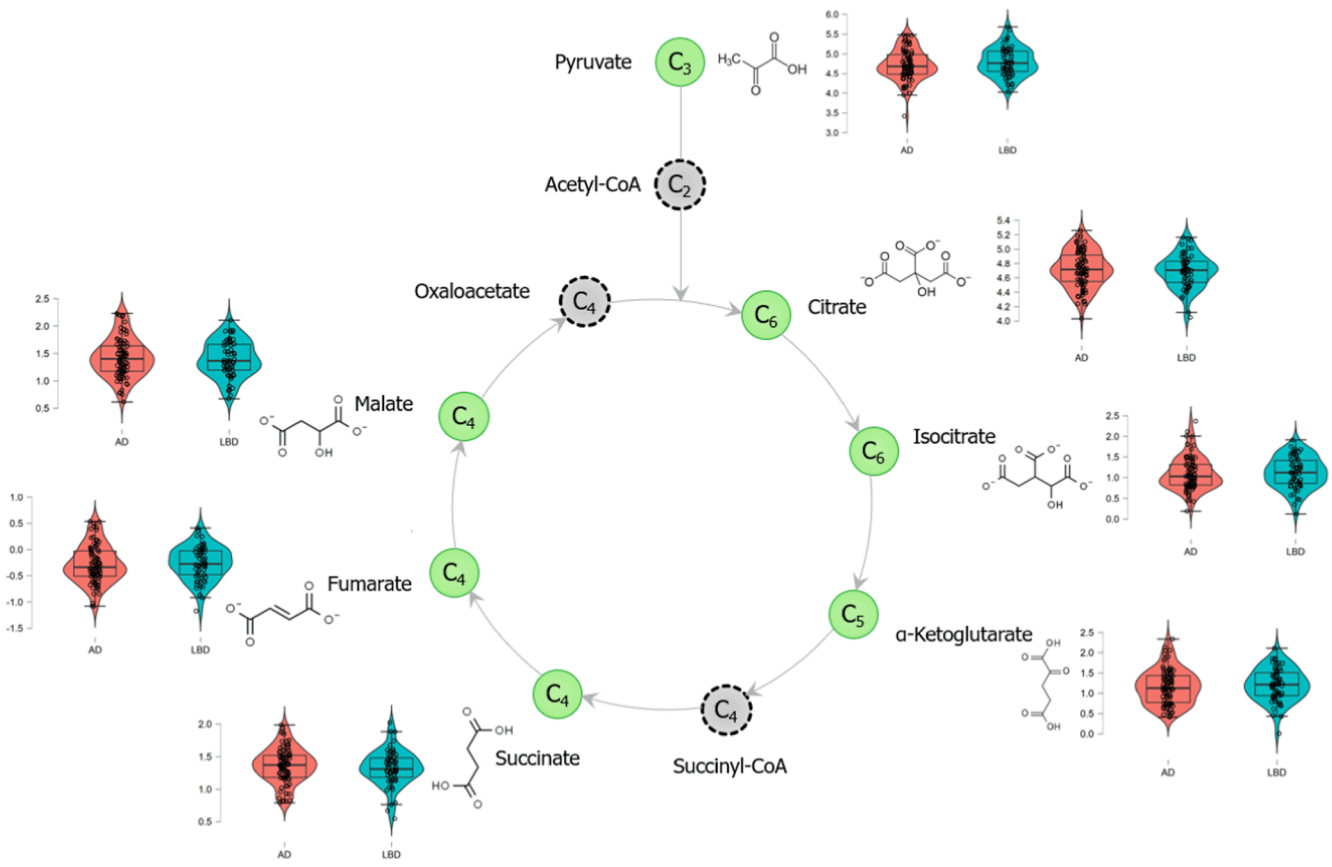

Fig. 1. Quantified TCA cycle metabolite concentrations in LBD and AD subgroups. LBD: Lewy Bodies Dementia (blue); AD: Alzheimer's Disease (red).Green circles depict quantified metabolites while gray circles represent non-measured metabolites within the TCA cycle. Violin plots show standardized log-transformed concentrations by each diagnosis subgroup. No significant group differences were found (p - values > 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

magnitude of the correlation coefficients was lower in the LBD subgroup for all the significant edges when compared to the AD network, although statistically significant group differences were not achieved, as explained in the Methods section (data not shown). Descriptive details about network centrality metrics in the total sample, LBD, and AD subgroups are presented in Supplementary Figs. 4 and 5.

Network models for the APOE-ε4 carriers are presented in Fig. 2B. We observed a variation in network topology (i.e., apparently reduced network connectivity) when models were conducted by APOE-ε4 subgroups (instead of LBD and AD subgroups). Most edges weights' estimations seem to be larger in APOE-ε4 non-carriers, particularly Pyruvate – Succinate (APOE-ε4 negative = −0.55; APOE-ε4 positive = −0.35), Succinate - Isocitrate (APOE-ε4 negative = 0.39; APOE-ε4 positive = 0.3), and Fumarate - Malate (APOE-ε4 negative = 0.74; APOE-ε4 positive = 0.71) with independency of α-ketoglutarate. Details about network topology and centrality estimations by APOE-ε4 allele are presented in Supplementary Fig. 5.

3.2. Tca cycle metabolites association with cognitive decline

Table 2 presents the results of both adjusted and unadjusted mixed-effects models using each metabolite as a predictor of cognitive performance at baseline (intercept column) and cognitive decline across study time points (slope column). After correcting the results for multiple testing, significant slope estimations were observed for citrate in the unadjusted model (FE = 0.79, 95 % CI = 0.36–1.21, p-value = <0.001, q-value = 0.008). This remained significant after adjusting for potential confounders (FE = 0.73, 95 % CI = 0.30–1.16, p-value = 0.001, q-value = 0.014). Therefore, as shown in Fig. 2C, the lower the citrate concentrations, the lower the MMSE scores in a 5-years follow-up.

Interactions by diagnosis (LBD and AD) were evaluated, but no significant effects were found (See Supplementary Fig. 6).

Table 3 and Fig. 2D shows adjusted model predictions for TCA cycle metabolites as predictors of cognitive decline by the APOE-ε4 subgroups. Across the 5-year follow-up, citrate (FE = 1.04, 95 % CI = 0.49–1.58, p-value = <0.001) and isocitrate (FE = 1.05, 95 % CI = 0.50–1.60, p-value = <0.001) concentrations were positively associated with MMSE scores only in carriers of any APOE-ε4 allele. However, a significant metabolite by APOE-ε4 interaction was observed exclusively for Isocitrate (p-interaction = 0.035). Thus, particularly in APOE-ε4 carriers, the lower the isocitrate concentration, the more rapid the decline of MMSE scores. Table 3 and Fig. 2D.

4. Discussion

This study aimed to analyze the potential associations of TCA cycle metabolites in serum and the rate of cognitive decline over five years, as well as to describe the TCA cycle metabolites networks in people with mild AD and LBD. Concentrations of citrate were associated with the rate of cognitive decline in both LBD and AD patients. Besides, isocitrate levels were associated with cognitive decline, particularly in carriers of an APOE-ε4 allele. Finally, the results of our network models are consistent with biochemical pathway findings in postmortem tissues (i.e., downregulation of the first half of the TCA cycle enzymes, with upregulation of the last half) (Atamna and Frey, 2007; Bubber et al., 2005; Kim et al., 1988; Rex Sheu et al., 1985; Sheu et al., 1994).

As metabolites are the end-point of enzyme reactions, thus, the

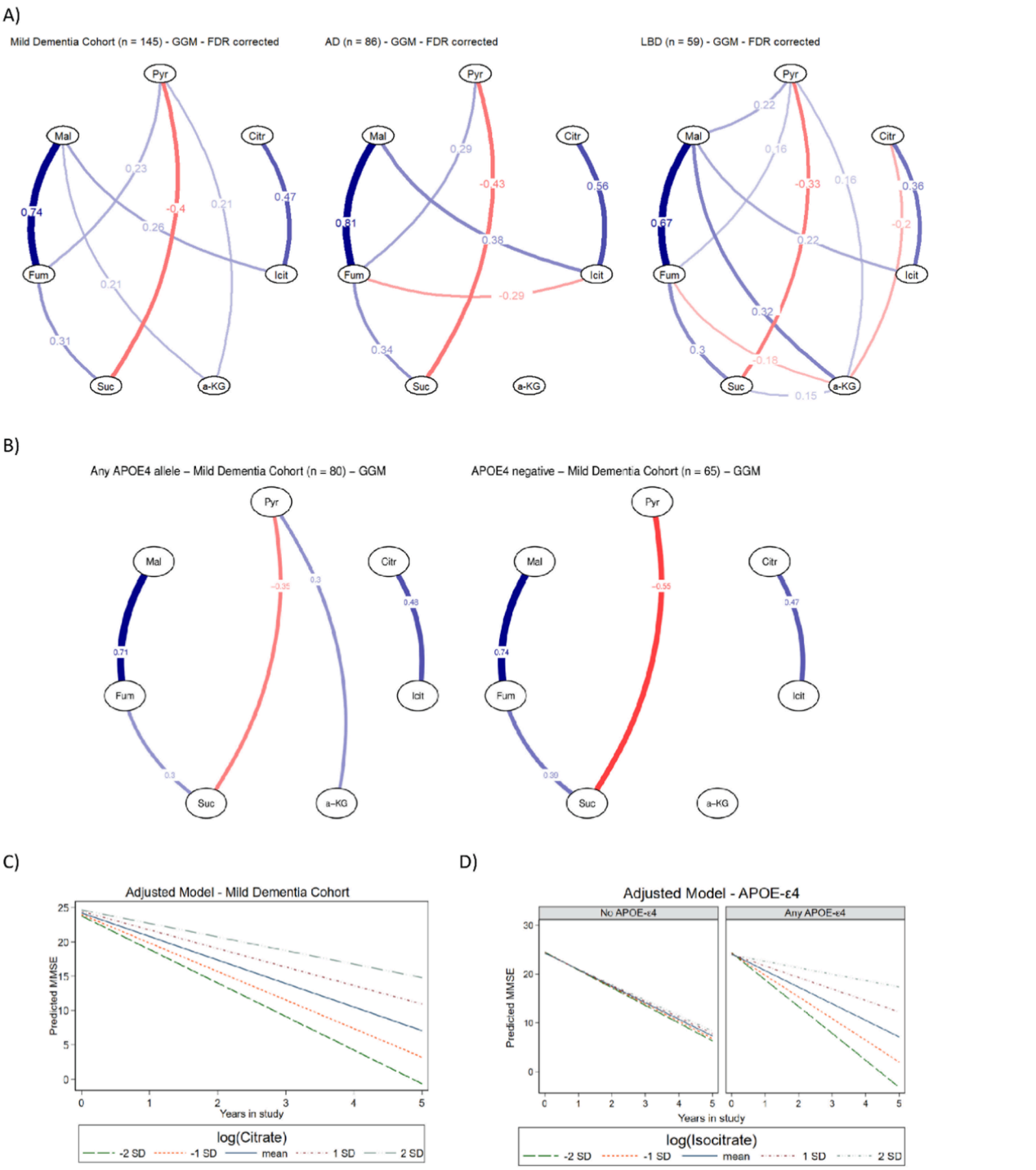

Fig. 2. Network analysis of TCA cycle metabolites and their effect on cognitive decline Pyr: Pyruvate; Cit: Citrate; Icit: Isocitrate; a-KG: α-ketoglutarate; Suc: Succinate; Fum: Fumarate; Mal: Malate; GM – FDR: Gaussian Graphical Modelling – False Discovery Ratio corrected; AD: Alzheimer's Disease; LBD: Lewy Bodies Dementia; MMSE: Mini-mental State Examination. APOE: Apolipoprotein E. SD: Standard Deviation. (A, B) The TCA cycle metabolites were plotted as nodes within each network model (i.e., Mild dementia cohort, Alzheimer's Disease, LBD, Negative APOE- ε4, and Positive APOE- ε4 subgroups). Depicted edges have FDR corrected q values < 0.05. Blue lines indicate positive partial correlations between a pair of metabolites, while red lines indicate negative partial correlations. The correlation coefficient was presented on each edge. (C) Adjusted Mixed-effects model estimations for baseline citrate concentration as a predictor of MMSE score over a 5-year follow-up in the mild dementia cohort (adjusted for age, sex, diagnosis, APOE status, and time, as well as interactions). The lower the baseline citrate concentrations, the lower the predicted cognitive decline over five years. (D) Adjusted Mixed-effects Tobit model with MMSE as the outcome. TCA cycle metabolite, time, and time*metabolite interaction per any APOE-ε4 allele using the Huber-White sandwich estimator to obtain robust standard errors. Plots show the prediction of longitudinal scores of MMSE by isocitrate baseline concentrations in APOE-ε4 non-carriers (left) and carriers of any ε4 allele (right). Significant associations between baseline isocitrate concentration and longitudinal MMSE score were observed only in those with any APOE-ε4 allele. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Associations of TCA cycle metabolite concentrations and cognitive decline over 5 years follow-up.

Unadjusted Models								
Metabolites	Intercept				Slope			
	FE	95 % CI	p-value	q-value	FE	95 % CI	p-value	q-value
Pyruvate	0.23	−0.22, 0.69	0.309	0.433	0.34	−0.19, 0.87	0.214	0.399
Citrate	0.15	−0.35, 0.64	0.559	0.652	0.79	0.36, 1.21	**<0.001**	**0.008**
Isocitrate	−0.08	−0.65, 0.48	0.780	0.870	0.63	0.17, 1.0.9	**0.007**	0.063
α-ketoglutarate	−0.29	−0.81, 0.22	0.266	0.433	0.41	−0.02, 0.84	0.060	0.210
Succinate	0.03	−0.46, 0.51	0.911	0.945	0.47	0.04, 0.90	**0.033**	0.185
Fumarate	0.31	−0.21, 0.82	0.244	0.427	0.36	−0.09, 0.81	0.120	0.373
Malate	0.28	−0.25, 0.81	0.296	0.433	0.44	−0.01, 0.89	0.058	0.210

Adjusted Models								
Metabolites	**Intercept**				**Slope**			
	FE	**95 % CI**	**p-value**	**q-value**	**FE**	**95 % CI**	**p-value**	**q-value**
Pyruvate	0.34	−0.16, 0.83	0.181	0.390	0.21	−0.32, 0.75	0.437	0.532
Citrate	0.22	−0.29, 0.73	0.395	0.503	0.73	0.30, 1.16	**0.001**	**0.014**
Isocitrate	−0.01	−0.57, 0.55	0.975	0.975	0.56	0.11, 1.00	**0.014**	0.098
α-ketoglutarate	−0.23	−0.75, 0.28	0.374	0.499	0.30	−0.13, 0.74	0.172	0.390
Succinate	0.06	−0.43, 0.55	0.808	0.870	0.45	0.01, 0.90	**0.045**	0.210
Fumarate	0.37	−0.14, 0.90	0.155	0.389	0.26	−0.21, 0.72	0.283	0.432
Malate	0.35	−0.19, 0.89	0.199	0.398	0.32	−0.13, 0.77	0.166	0.390

FE: Fixed Effects. CI: Confidence Interval.
Mixed-effects Tobit model with MMSE as the outcome. Each metabolite was used as the predictor, and time, age, gender, APOE ε4, and diagnosis as covariates (as well as significant time interactions). The Huber-White sandwich estimator was used to obtain robust standard errors. The models were conducted in the total sample.
The intercept column show model estimations for time = 0 (baseline), while the slope column shows longitudinal model estimations (time = 1 to 5 years) for cognitive decline as the outcome.
q-values = p-values adjusted for multiple comparisons according to Benjamini Hochberg (False Discovery Ratio) method (all adjusted and unadjusted associations with intercept and slope constitute m number of tests).
P and q - values < 0.05 are printed in bold.

Table 3
APOE-ε4 interaction effect over the associations of TCA cycle metabolite concentrations and cognitive decline over 5 years follow-up.

		Intercept				Slope			
Metabolites	APOE-ε4 status	FE	95 % CI	p-value	p-int	FE	95 % CI	p-value	p-int
Pyruvate	APOE-ε4	0.29	−0.32, 0.90	0.356	0.729	0.15	−0.56, 0.86	0.682	0.522
	No APOE-ε4	0.11	−0.60, 08.2	0.762		0.45	−0.28, 1.17	0.226	
Citrate	APOE-ε4	0.13	−0.60, 0.85	0.729	0.996	1.04	0.49, 1.58	**< 0.001**	0.348
	No APOE-ε4	0.14	−0.53, 0.80	0.686		0.59	−0.07, 1.26	0.082	
Isocitrate	APOE-ε4	−0.11	−0.85, 0.63	0.771	0.952	1.05	0.50, 1.60	**< 0.001**	**0.035**
	No APOE-ε4	−0.08	−0.96, 0.81	0.886		0.08	−0.62, 0.77	0.828	
α-ketoglutarate	APOE-ε4	−0.41	−1.16, 0.33	0.280	0.777	0.55	−0.01, 1.11	0.055	0.611
	No APOE-ε4	−0.27	−0.99, 0.45	0.465		0.30	−0.36, 0.96	0.369	
Succinate	APOE-ε4	−0.37	−1.02, 0.29	0.274	0.151	0.47	−0,14, 1.07	0.130	0.658
	No APOE-ε4	0.35	−0.36, 1.05	0.338		0.65	−0.01, 1.31	0.055	
Fumarate	APOE-ε4	0.16	−0.69, 1.01	0.710	0.650	0.36	−0.23, 0.96	0.233	0.985
	No APOE-ε4	0.39	−0.23, 1.02	0.220		0.34	−0.34, 1.03	0.328	
Malate	APOE-ε4	0.02	−0.79, 0.83	0.960	0.406	0.55	−0.06, 1.16	0.080	0.630
	No APOE-ε4	0.47	−0.22, 1.15	0.181		0.30	−0.35, 0.95	0.363	

FE: Fixed Effects. CI: Confidence Interval. APOE: Apolipoprotein E. p-int: p-value for metabolite interaction by any APOE-ε4 allele.
Mixed-effects Tobit model with MMSE as the outcome. TCA cycle metabolite, time, and time*metabolite interaction per any APOE-ε4 allele (including interactions) using the Huber-White sandwich estimator to obtain robust standard errors.
The intercept column show model estimations for time = 0 (baseline), while the slope column shows longitudinal model estimations (time = 1 to 5 years) for cognitive decline as the outcome.
P values < 0.05 are printed in bold.

closest point to the phenotype, metabolomic methods such as network analysis can indirectly reflect the whole biological profile facilitating a mechanistic insight (Tsuruoka et al., 2013; Perez De Souza et al., 2020; Johnson et al., 2016), showing the organization of metabolically functional modules (i.e., highly connected metabolites) (Fukushima et al., 2011). Results of our network models consistently showed negative partial correlations between pyruvate - succinate, as well as positive partial correlations between fumarate – malate and citrate - Isocitrate. The above pattern was observed in both LBD and AD subgroups, as well as in APOE-ε4 carriers and non-carriers. Thus, this dementia-related connectivity pattern might not depend on diagnosis or APOE-ε4 genotype.

Preliminary findings in human enzymatic activities in AD patients are summarized in Fig. 3. The consistently reported downregulation of pyruvate dehydrogenase complex (PDHC), citrate synthase (CS), isocitrate dehydrogenase (IDHC), and α-ketoglutarate dehydrogenase complex (α-KDHC) could be evidenced in our models. Thus, the moderate-to-high negative partial correlation between pyruvate and succinate might indirectly reflect a reduction in effective enzymatic activity for substrate-to-product conversion in various TCA cycle steps

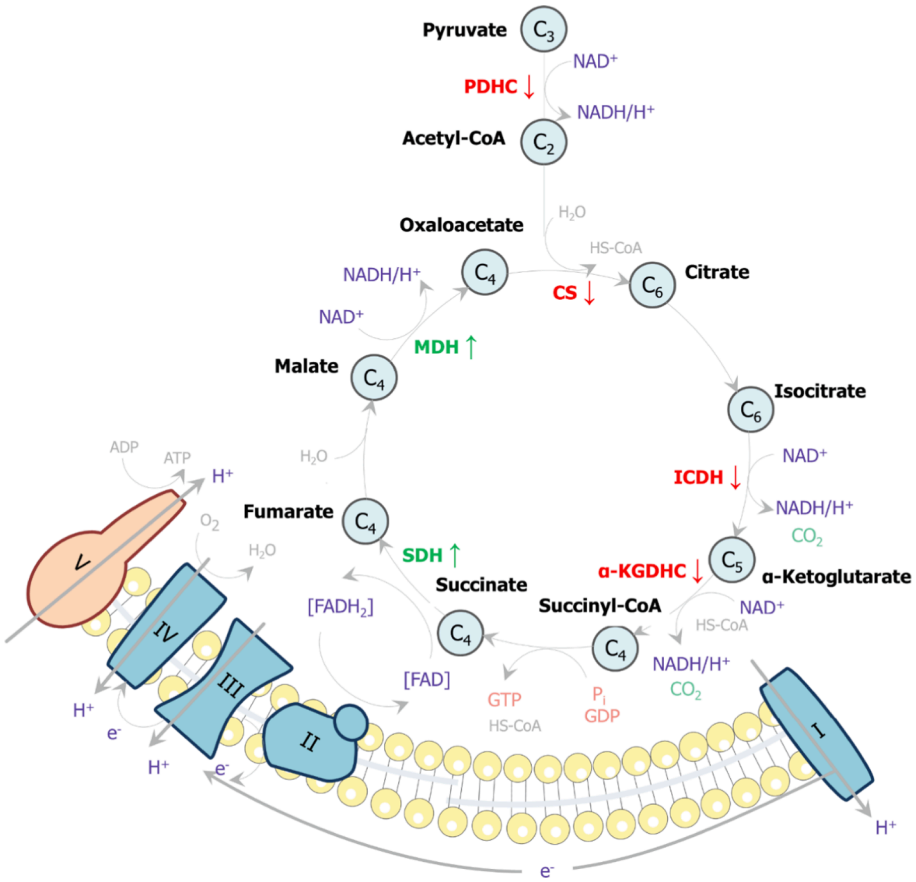

Fig. 3. Graphical summary of reported abnormalities of TCA cycle enzymatic activity in AD Reduced activity in bold red enzymes with upregulation of bold green enzymes has been reported in AD patients (Bubber et al., 2005). Note the number of carbons in each intermediate metabolite; enzymes with reduced activity have both dehydrogenase and decarboxylase functions, whereas the upregulated enzymes have only dehydrogenase activity. PDHC: Pyruvate dehydrogenase complex. CS: Citrate synthase. ICDH: Isocitrate dehydrogenase. α-KDHC: α-ketoglutarate dehydrogenase complex. SDH: Succinyl dehydrogenase. MDH: Malate dehydrogenase. NAD^+/NADH: Nicotinamide adenine dinucleotide. GTP: Guanosine triphosphate. Pi-GDP: Inorganic phosphate-guanosine diphosphate. FAD/ $FADH_2$: Flavin adenine dinucleotide. I: NADH dehydrogenase (respiratory Complex I), electron transport chain. II: Succinate dehydrogenase (respiratory Complex II), electron transport chain. III: Respiratory Complex III, electron transport chain. IV: Respiratory Complex IV, electron transport chain. V: ATP synthase (Respiratory Complex V), electron transport chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from pyruvate to succinate (i.e., downregulation of the first half of TCA cycle). By contrast, we have observed the strongest effect size for the positive partial correlations between fumarate and malate, and we hypothesize that this finding might indirectly reflect upregulated enzymes in dementia patients, namely succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) (Bubber et al., 2005; Yan et al., 2020). This hypothesis should be tested in future studies of metabolite-metabolite networks in older adults in order to control for potential age-related effects.

Although the underlying mechanism of these TCA cycle dysregulation has not been elucidated yet, it has been noted that the downregulated enzymes have both dehydrogenase and decarboxylase activity (Bubber et al., 2005; Wang et al., 2020), whereas upregulated enzymes have only dehydrogenase properties (Bubber et al., 2005; Yan et al., 2020). Another potential rationale for our findings comes from *in vitro* studies in inflammation: it has been demonstrated that activated dendritic cells have an abnormal TCA cycle which leads to the accumulation of citrate and succinate. Thereby, mitochondrial citrate can inhibit both PDHC and SDH, resulting in aberrant nuclear and inflammatory processes, but the clinical implications of this phenomenon have not been elucidated yet (Williams and O'Neill, 2018).

Preliminary publications have examined enzymatic activity in various tissues, such as postmortem brains (Bubber et al., 2005), fibroblasts from the skin (Sheu et al., 1994), and lymphocytes and platelets from AD patients (Wilkins et al., 2017; Wilkins et al., 2021). However, studies in serum samples are still scarce despite valid methods for operationalized TCA cycle serum metabolite quantification and their promising use as potential metabolomic biomarkers in other diseases (Rathod et al., 2020; Martínez-Reyes and Chandel, 2020; Hallan et al., 2017). To the best of our knowledge, only one pilot study with serum and saliva samples aimed to quantify TCA cycle dysfunction only in a small heterogeneous group of patients with different neurodegenerative dementias. This study showed increased isocitrate concentration in dementia patients compared to age-matched controls (Tsuruoka et al., 2013). Therefore, quantification of TCA cycle metabolites concentration in serum may indirectly display dementia-related mitochondrial abnormalities in energy metabolism. However, few studies have used network analysis for metabolomic analysis in dementia, and further investigations should integrate the TCA cycle metabolites into broader networks (such as fatty acids) in order to examine interactions between this and other metabolic pathways in people with neurodegenerative diseases and older adults.

Our results suggest that concentrations of citrate (and Isocitrate in APOE-ε4 carriers) can predict subsequent cognitive decline in people with mild AD and LBD. *In-vivo* evidence investigating blood-based biomarkers for longitudinal cognitive performance is scarce, particularly in LBD subjects and APOE-ε4 carriers. However, in older adults and patients with AD, the plasmatic concentration of citrate has been linked to hippocampal and whole-brain atrophy (de Leeuw et al., 2021). Similarly, in serum, a previous study has shown associations between high citrate concentration and increased risk of AD (González-Domínguez et al., 2015). However, we are not aware of any other publications suggesting serum citrate as an early biomarker of cognitive prognosis in people with dementia, and evidence is not conclusive regarding the direction of the effect of TCA cycle metabolites over cognition. Therefore, we encourage further research to examine the reproducibility of our findings and external validity in other populations, as well as to control for specific confounders of serum metabolite concentrations in the study protocols.

On the other hand, a very recent secondary analysis of the Study of Latinos-Investigation of Neurocognitive Aging (SOL-INCA) found a significant positive association between the serum isocitrate concentration and the risk of MCI diagnosis in healthy older people (He et al., 2022). We found a significant effect of Isocitrate over MCI risk was observed

exclusively in APOE-ε4 carriers but in the opposite direction (i.e., higher concentrations associated with slower cognitive decline). Regardless, the 7-years global cognitive decline rate serum in that sample was not significantly associated with isocitrate concentration. The latter could be explained by differences in the neuropathological burden (not present in all MCI patients), clinical heterogeneity, variability of metabolite concentrations, psychometric instruments, or the methods used for modeling the cognitive outcome. Thus, to study the longitudinal cognitive decline, we used longitudinal mixed-effects Tobit models taking into account the ceiling effects of MMSE scores in the early stage of dementia, as well as floor effects observed in severe dementia (Giil et al., 2021; Twisk and Rijmen, 2009).

Whereas multiple animal models have suggested an important role of α-ketoglutarate in histone methylation (Sharma and Ramanathan, 2020), human studies have reported potential confounders such as sex and age in α-ketoglutarate plasma concentration (Lawton et al., 2008). More insights come from the Framingham Offspring Study cohort; their results suggested that increased plasmatic isocitrate and malate concentrations were negatively associated with longevity (Cheng et al., 2015). Furthermore, in older adults, serum levels of pyruvate, Isocitrate, malate, fumarate, *cis*-aconitate, and glucuronate seem to be associated with increased frailty (Pan et al., 2022). Future works examining serum TCA cycle metabolites as potential early markers of cognitive decline and other relevant outcomes for older adult patients with dementia (such as frailty) are needed to unravel the contribution of mitochondrial abnormalities in dementia pathophysiology and clinical manifestations.

There are some limitations of this study to be considered. We emphasize that our network estimations are not a direct measurement of enzymatic activity/expression. Serum concentrations of TCA cycle metabolites might not reflect intracellular abnormalities and may also depend on intake and substrate bioavailability, as well as demographic confounders. Thus, specific designs analyzing the contribution of enzyme activity/expression over the concentration of TCA cycle metabolites (and networks) are still needed to formally confirm our hypothesis. Also, serial samples over the study time points would provide valuable information about the progression of these potential markers throughout the dementia course, clarifying the direction of the effects of metabolite concentrations over cognitive decline. Other potential limitations include a potential selection bias (patients with the more severe or atypical course may be more likely to be referred), the relatively low sample size, the relatively high dropout rate (particularly in the LBD group due to death), and the lack of a normal control group. Finally, as an important limitation, this exploratory work could not evaluate the confounder effects of different comorbidities. We encourage further efforts to assess this important factor in larger populations or patients with specific comorbidities that might play a confounder effect. As a case in point, one study showed increased isocitrate and Fumarate plasmatic concentrations in nondiabetic patients with Chronic kidney disease in stages 3 – 4 (Hallan et al., 2017). In addition, physical activity and exercise can modulate TCA cycle plasmatic concentrations in metabolites such as citrate, succinate, fumarate, and malate are known to be released. In line with the above, malate plasmatic concentrations and *cis*-aconitate can differentiate frail and non-frail older adults and young subjects, showing a potential age-related increase of alpha-ketoglutarate, succinate, and fumarate (Westbrook et al., 2022).

5. Conclusions

In patients with mild dementia due to LBD and AD, serum citrate in the early stage of dementia might predict cognitive prognosis, as well as Isocitrate in APOE-ε4 carriers. In addition, in both LBD and AD, down-regulation of biochemical pathways in the first half of the TCA cycle (decarboxylating dehydrogenases) combined with upregulation in the latter half (dehydrogenases only), could be indirectly reflected by serum TCA cycle metabolites using network analyses. Further studies are needed to provide external validity to our conclusions.

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CRediT authorship contribution statement

Alberto Jaramillo-Jimenez: Visualization, Formal analysis, Conceptualization, Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Lasse M. Giil:** Formal analysis, Methodology, Supervision, Writing - review & editing. **Diego A. Tovar-Rios:** Formal analysis, Methodology, Data curation, Writing - review & editing. **Kåre Andre Kristiansen:** Methodology, Data curation, Writing - review & editing. **Per Bruheim:** Methodology, Data curation, Writing - review & editing. **Dag Aarsland:** Supervision, Methodology, Conceptualization, Funding acquisition, Project administration, Writing - review & editing. **George E. Barreto:** Supervision, Methodology, Conceptualization, Investigation, Writing - review & editing. **Rolf Kristian Berge:** Supervision, Methodology, Data curation, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2023.05.002.

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