Combined Metabolic Activators Improve Cognitive Functions in Alzheimer’s Disease Patients: A Randomised, Double-Blinded, Placebo-Controlled Phase-II Trial

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Abstract

Background

Alzheimer's disease (AD) is associated with metabolic abnormalities linked to critical elements of neurodegeneration. We recently administrated Combined Metabolic Activators (CMA) to the AD rat model and observed that administration of CMA activated the mitochondrial functions and eventually improved the AD-associated histological parameters in the animals. CMA consists of NAD + and glutathione precursors and includes L-serine, nicotinamide riboside, N-acetyl-L-cysteine, and L-carnitine tartrate.

Methods

Here, we designed a randomised, double-blinded, placebo-controlled phase-II trial and studied the effect of CMA administration on the global metabolism of AD patients. The primary endpoint was on the difference in cognitive and daily living activity scores between the placebo and the treatment arms. The secondary aim of this study was to evaluate the safety and tolerability of CMA. We also performed a comprehensive human plasma metabolome and proteome analysis.

Results

Based on our analysis, we showed a significant decrease of ADAS-Cog scores on Day 84 vs Day 0 (p = 0.00001, 29% improvement) in the CMA group. Moreover, there was a significant enhancement (p = 0.0073) in ADAS-Cog scores between CMA and placebo groups in patients with higher ADAS-Cog scores. Improved cognitive functions were endorsed with relevant hippocampal volumes and cortical thickness alterations. Moreover, the plasma levels of proteins and metabolites associated with NAD + and glutathione metabolism are significantly improved after treatment.

Conclusion

In conclusion, our results show that treating AD patients with CMA leads to enhanced cognitive functions associated with the improved metabolome, proteome and structural neuroimaging parameters, suggesting a role for such a therapeutic regimen in treating patients, especially with severe AD.

Trial registration:


Background
Alzheimer's disease (AD) is characterised by progressive synaptic and axonal dysfunction, neuronal loss and cognitive decline (1). There is growing evidence that AD is closely associated with metabolic and oxidative stress linked to critical elements of neurodegeneration, such as mitochondrial dysfunctions and bioenergetic impairments (2, 3). Indeed, increasing data indicate that systemic metabolic disorders, such as insulin resistance, are strongly associated with bioenergetic failure of nerve cells (4, 5). This can manifest as cognitive impairment and brain-specific neuropathology while sharing common pathogenic mechanisms with AD, such as impaired glucose metabolism, oxidative stress, insulin resistance, and amyloidogenesis (4, 6, 7). Recent evidence accordingly suggests that patients with type 2 diabetes mellitus are at increased risk of developing AD (6).

Although the disease is defined by the accumulation of abnormal amyloid and tau proteins (8), the mechanistic assumption of linear causality between the amyloid cascade and cognitive dysfunction in AD is still lacking, since amyloid-lowering approaches have failed to provide cognitive benefits in human clinical trials (9). A growing body of evidence suggests that impaired brain energy metabolism and mitochondrial dysfunction in AD may contribute to cognitive decline. At the same time, therapeutic options, such as drugs typically prescribed for metabolic disorders that improve metabolic status, may slow cognitive decline or prevent dementia progression (10). This is suggested by positron emission tomography imaging studies revealing baseline cerebral glucose metabolism abnormalities before the onset of cognitive symptoms in patients with AD (11). In addition, recent preclinical data indicate that ageing and AD are associated with the reorganisation of brain energy metabolism and mitochondrial dysfunction, including an overall increase in lactate secretion and the downregulation of bioenergetic enzymes (12, 13).

A divergent approach combining multiple compounds that simultaneously reduce oxidative injury and improve bioenergetics, in other words, targeting various pathways has been proposed as a therapeutic strategy associated more likely with successful translational outcomes (14). Previous research identified limited serine availability, reduced de novo glutathione synthesis, and altered NAD+ metabolism based on the combining multi-omics profiling of the transgenic mouse model of AD (15). Consistent with this, it also has been reported that age- and AD-associated metabolic shifts responded well to NAD(P)+/NAD(P)H redox-dependent reactions in mice (16, 17). These findings were confirmed by human metabolomic data showing significantly altered cerebrospinal fluid acylcarnitine levels in patients with AD, which correlated with the decline of cognitive functions and structural brain abnormalities (18, 19).

In addition to the above-mentioned metabolic underpinnings of AD, several neuroimaging studies have indicated that AD is characterised by critical cognitive regional alterations, notably including the hippocampal volumes and cortical thickness, including especially inferior parietal, middle frontal and occipital regions (20). For instance, Nagata et al. showed that the vulnerability of the hippocampus plays a potential role in memory and executive dysfunction in AD (21, 22). Similarly, several neuroimaging studies showed that cortical thickness plays a critical role in AD pathophysiology (23, 24). Despite these promising studies, no research has evaluated fundamental common pathophysiological mechanisms shared by systemic metabolic alterations and specific brain areas involved in cognitive deterioration in
AD. For instance, whether metabolic stimulation acts on the systems biology and brain structural level in AD patients remains an enigma. Systems-biology-based approaches focusing on regional brain correlates of altered systemic metabolism in AD is required to overcome such translational limitations.

Based on integrative network analysis of multi-omics data of non-alcoholic fatty liver disease, we have developed the combined metabolic activators (CMA) consisting of L-serine, N-acetyl cysteine (NAC), nicotinamide riboside (NR), and L-carnitine tartrate (LCAT, the salt form of L-carnitine) and showed that administration of CMA activates mitochondria, improves inflammation markers in animals and humans (25–29). We have also found that the CMA administration promotes mitochondrial fatty acid uptake from the cytosol, facilitates fatty acid oxidation in the mitochondria, and alleviates oxidative stress (30). Recently, we reported that CMA administration effectively increased fatty acid oxidation and de novo glutathione generation, as evidenced by metabolomic and proteomic profiling (25). Moreover, plasma levels of metabolites associated with antioxidant metabolism and inflammatory proteins were improved in COVID-19 patients treated with CMA compared to the placebo (29).

Based on these studies, we hypothesized that CMA administration may activate the mitochondria and improve brain metabolism in AD patients. Here, we designed a randomised, double-blinded, placebo-controlled human phase 2 clinical study and studied the effect of CMA administration on the global metabolism of AD patients by comprehensive metabolomics and proteomics analysis.

**Material And Methods**

**Clinical Trial Design and Oversight**

Patients for this randomised, parallel group, two-arm, double-blinded, placebo-controlled, phase 2 study were recruited at the Faculty of Medicine, Alanya Alaaddin Keykubat University, Antalya, Turkey and Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey. Written informed consent was obtained from all participants before initiating any trial-related procedures. An independent external data-monitoring committee oversaw the safety of the participants and the risk-benefit analysis. The trial was conducted following Good Clinical Practice guidelines and the principles of the Declaration of Helsinki. The ethics committee approved the study of Istanbul Medipol University, Istanbul, Turkey (Date:22.01.2020, Decision No: 7), and registered at https://clinicaltrials.gov/ with Clinical Trial ID: NCT04044131.

**Eligibility criteria of clinical trial participants**

Patients were enrolled in the trial if they were over 50 years of age with mild to moderate AD according to ADAS-cog (AD Assessment Scale-cognitive subscale; ADAS ≥ 12) and the Clinical Dementia Rating Scale Sum of Boxes (CDR-SOB; CDR ≤ 2). Patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders-5 diagnostic criteria. Patients who had a history of stroke, severe brain trauma, and toxic drug exposure were excluded. The main characteristics of the patients are summarised
in Dataset S1. The inclusion, exclusion, and randomisation criteria are described in detail in the Supplementary Appendix.

**Randomisation, Interventions, and Follow-up**

Patients were randomly assigned to receive CMA or placebo (2:1). Patient information (patient number, date of birth, initials) was entered into the web-based randomisation system, and the randomisation codes were entered into the electronic case report form. All clinical staff were blinded to treatment, as were the participants.

Treatment started on the day of diagnosis. Both placebo and CMA were provided in powdered form in identical plastic bottles containing a single dose to be dissolved in water and taken orally, one dose in the morning after breakfast and one dose in the evening after dinner. Each dose of CMA contained 3.73 g L-carnitine tartrate, 2.55 g N-acetylcysteine, 1 g nicotinamide riboside chloride, and 12.35 g serine. All patients received one dose during the first 28 days and two doses until Day 84. All patients came for a follow-up visit on Day 84. Further information is provided in the study protocol (Supplementary Appendix).

**Outcomes**

The primary endpoint in the original protocol was to assess the clinical efficacy of CMA in AD patients. For the primary purpose, the clinical differences in cognition of subjects receiving twelve-week treatment either with metabolic activators supplementation or placebo were determined. The primary analysis was on the difference in cognitive and daily living activity scores between the placebo and the treatment arms, which were assessed by Mini-Mental State Examination (MMSE), AD Assessment Scale-cognitive subscale (ADAS-Cog) and AD Cooperative Study - Activities of Daily Living (ADCS-ADL) in AD patients. The secondary aim of this study was to evaluate the safety and tolerability of CMA. All protocol amendments were authorised and approved by the sponsor, the institutional review board, the independent ethics committee, and the pertinent regulatory authorities. Sample size is estimated by statistical power analysis (Supplementary appendix).

The number and characteristics of adverse events, serious adverse events, and treatment discontinuation due to CMA were reported as key safety endpoints from the beginning of the study to the end of the follow-up period. The changes in vital signs, baseline values, and treatment status were recorded on Days 0 and 84. A complete list of the endpoints is provided in the Supplementary Appendix.

**Proteomics Analysis**

Plasma levels of proteins were determined with the Olink panel (Olink Bioscience, Uppsala, Sweden). Briefly, each sample was incubated with DNA-labelled antibody pairs (proximity probes). When an antibody pair binds to its corresponding antigens, the corresponding DNA tails form an amplicon by proximity extension, which can be quantified by high-throughput, real-time PCR. Probe solution (3 µl) was mixed with 1 µl of sample and incubated overnight at 4°C. Then 96 µl of extension solution containing extension enzyme and PCR reagents for the pre-amplification step was added. The extension products
were mixed with detection reagents and primers and loaded on the chip for qPCR analysis with the BioMark HD System (Fluidigm Corporation, South San Francisco, CA). To minimise inter- and intrarun variation, the data were normalised to both internal and interplate control. Normalised data were expressed in arbitrary units (Normalized Protein eXpression, NPX) on a log2 scale and linearised with the formula 2NPX. A high NPX indicates a high protein concentration. The limit of detection, determined for each of the assays, was defined as three standard deviations above the negative control (background).

**Untargeted Metabolomics Analysis**

Plasma samples were collected on Days 0 and 84 for nontargeted metabolite profiling by Metabolon (Durham, NC). The samples were prepared with an automated system (MicroLab STAR, Hamilton Company, Reno, NV). For quality control purposes, a recovery standard was added before the first step of the extraction. To remove protein and dissociated small molecules bound to protein or trapped in the precipitated protein matrix and recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min and centrifuged. The resulting extract was divided into four fractions: one each for analysis by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with positive ion-mode electrospray ionisation, UPLC-MS/MS with negative ion-mode electrospray ionisation, and gas chromatography-mass spectrometry; one fraction was reserved as a backup.

**Determination of clinical variables informing response to CMA administration**

The patient groups with low and high levels of each clinical parameter were established based on the median score for that clinical parameter across all patients on Day 0. Patients scoring at or below the median were placed in the low group; patients scoring above the median were placed in the high group. ADAS-Cog scores were measured over different time points, and statistical significance was tested between time points by using a paired t-test. Clinical parameters were deemed informative for the response to CMA if precisely one group (low or high) exhibited more statistically significant changes in ADAS-Cog in the CMA group than in the placebo group.

**MRI recording parameters and analysis**

Among the entire patient cohort, 40 MRI-compatible patients, 29 in the CMA group and 11 in the placebo group, were recruited for the structural MRI study. Structural magnetic resonance brain imaging was recorded in the 1.5 T SIGNA Explorer MRI device with a 16-channel head coil (General Electric Company, United States).

Hippocampal subfield segmentation and grey/white matter volumetric segmentation were performed using Freesurfer image analysis suite 6.0 (stable6–20170118) and the integrated hippocampal subfield segmentation module, which is documented and freely available for download. All T1-weighted images were preprocessed with the standard Freesurfer processing pipeline using the “recon-all” script. In addition to the default processing pipeline, the high-resolution T2-weighted images of each participant
were submitted using the “hippocampal-subfield-T1” measure. The Freesurfer algorithm segments 12 hippocampal subfields: hippocampal tail, subiculum, CA1, hippocampal fissure, presubiculum, parasubiculum, molecular layer, granule cell layer of the DG, CA2–3, CA4, fimbria, and the hippocampal–amygdaloid transition area (HATA). The volume estimates of these subfields (combined for the right and left hemispheres of each subfield) were then used in the final analysis.

**Image processing**

To obtain hippocampal subfield measurements, each T1 image was processed using FreeSurfer version 7.1.0 (http://surfer.nmr.mgh.harvard.edu/). Each T1 image was processed using FreeSurfer version 7.1.0 (http://surfer.nmr.mgh.harvard.edu/). A Standard and automatic reconstruction algorithm was used to preprocess and the hippocampal subfield segmentation steps. The hippocampal subfield segmentation of each subject was visually inspected and determined to be free from errors by two independent researchers. Outliers of each subregion volume were defined as data more than 1.5 interquartile range below the first quartile or above the third quartile, and these data were included in the analyses. We used the composite subfield definitions based on the detailed segmentations performed by FreeSurfer and defined the anterior region as the sum of the regions CA1, CA3, CA4, molecular layer, granular cell layers of the dentate gyrus (GC/DG), subiculum, and presubiculum in the hippocampal head. The posterior region consisted of the same subregions in the hippocampal body and the hippocampal tail. The CA composite region was defined as the sum of the volumes of the CA1 region, CA3 region, subiculum, and molecular layer. The DG composite region included the CA4 region and the GC/DG. The subiculum composite region was defined as the presubiculum of the FreeSurfer subfields. The volume of each subfield was calculated separately in the anterior (head), and posterior (body) regions of the hippocampus and hippocampal brain volume was standardised by dividing each by the Intracranial Volume (ICV), giving intracranial volume–corrected regional brain volume data.

Cortical thickness was measured using the FreeSurfer image analysis suite (V6.0.0, http://surfer.nmr.mgh.harvard.edu/) by computing the averaged distance between the grey/white matter boundary and pial surface at each vertex on the cortical surface. Longitudinal analysis was performed using the FreeSurfer image analysis and FreeSurfer's longitudinal processing pipeline program (V6.0.0, http://surfer.nmr.mgh.harvard.edu/) using unbiased-subject specific T1 MRI scans for each subject. After several imaging processing steps (skull stripping, Talairach transformation, atlas registration, spherical surface maps and parcellations) based on the subject-specific templates, the cerebral cortex was parcellated into 68 distinct anatomical regions, the averaged thickness was determined, and each subject-specific map was visually analysed before further analysing steps.

**Statistical Analysis**

Paired t-test was used to identify the differences in clinical parameters between time points, and one-way ANOVA was used to find the shifts between CMA and placebo groups at each time point. Cohen's d effect size was estimated by R package “effsize” paired parameter was used when we compared difference between different visits. For analysis of plasma metabolomics, we removed the metabolite profiles with
more than 50% missing values across all samples. Metabolite changes between time points were analysed by paired t-test. Metabolite changes between CMA and placebo groups were analysed by one-way ANOVA. Missing values were removed in pairwise comparison. The p-values were adjusted by Benjamini & Hochberg method. Metabolites with a false-discovery rate of 5% were considered statistically significant.

For analysis of plasma proteomics, we removed the protein profiles with more than 50% missing values across all samples. A paired t-test was used to identify the changes between time points, and one-way ANOVA was used to determine the changes between different groups. P < 0.01 was considered statistically significant. Spearman correlation analysis was used to analyse the association between CMA and clinical parameters or metabolomics or proteomics.

We performed a Wilcoxon test on the hippocampal volume differences between the pre-post data for the structural MRI analysis to show the changes with the treatment effect. We shortly looked for significant interactions in the following areas: pre-and post-treatment group differences and time effects (pre-and post-treatment differences adjusted for active and placebo groups) as previously defined by Clarkson et al. 2018 (38). By using longitudinal analysis paradigm we created GLM design matrix for active and placebo groups consisting of a) CMA and placebo patients Day 0 and Day 84 images, b) baseline of Day 0 and Day 84 combined image and c) time difference (year). Similarly, for the cortical thickness analysis we used that the symmetrised percent change (spc) is the rate concerning the average thickness: spc = rate / avg (Reuter et al. 2012). We used positive and negative Monte Carlo simulation with the threshold of 1.3 to define significantly changed thickness areas (p < 0.05).

**Generation of Multi-Omics Network**

A multi-omics correlation network was generated based on all patients’ clinical parameters, serum chemistry, metabolomics, and proteomics data. We followed the multi-omics network generation pipeline from iNetModels (39). Spearman correlations between analytes were calculated using the SciPy package in Python 3.7. Missing values were removed pairwisely by setting the “nan_policy” variable to “omit”. Significant correlations (FDR < 5%) were kept and used to link analytes from the same and different omics. The downstream analysis, i.e. centrality analysis, was performed using the degree centrality calculation in iGraph Python package in Python 3.7.

**Results**

**CMA Improves Cognition and Blood Parameters in Alzheimer’s Disease Patients in a randomized double-blinded clinical trial**

To test the effect of the CMA in AD patients, we performed a double-blind, randomised, placebo-controlled phase 2 study and screened 89 adults diagnosed with AD. We recruited 69 patients older than 50 years with mild to moderate AD according to ADAS-Cog (AD Assessment Scale-cognitive subscale; ADAS ≥ 12) and the Clinical Dementia Rating Scale Sum of Boxes (CDR-SOB; CDR ≤ 2) between 01.02.2020–
01.10.2020. Of the 69 patients, 47 were randomly assigned to the CMA group and 22 to the placebo group and completed visit 2 after 28 days. Nine patients (7 in CMA, 2 in placebo groups) dropped out of the study before Day 84 visit during the COVID-19 lockdown. Of these patients, 60 (40 in the CMA group and 20 in the placebo group) completed visit 3 after 84 days (Fig. 1A, Figure S1). We assessed the clinical variables on Days 0, 28 and 84, and analysed the differences between the CMA and placebo groups (Dataset S1 and S2).

The patients’ mean age in the study was 70.8 years (56–86 years), and 52.1% were men (Table 1, Dataset S1). The mean ADAS-Cog score was 22.88 (± 10.51) for CMA and 26.28 (± 17.35) for placebo (Table 1, Dataset S2). There was no significant difference for the baseline levels of these demographic parameters and AD clinical indicators between CMA and placebo groups (Table 1). The other clinical characteristics were similar in the CMA and placebo groups (Dataset S1 and S2). Regarding safety, no severe adverse events occurred, and 5 patients (7.2%) reported adverse events. All decided to complete the study (Table 2).

### Table 1
Demographics and baseline characteristics of the study population*

<table>
<thead>
<tr>
<th></th>
<th>CMA (n = 47)</th>
<th>Placebo (n = 22)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>70.77 ± 8</td>
<td>70.91 ± 7.54</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25 (53.2%)</td>
<td>11 (50%)</td>
<td>0.88</td>
</tr>
<tr>
<td>Female</td>
<td>22 (46.8%)</td>
<td>11 (50%)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>Caucasian (100%)</td>
<td>Caucasian (100%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Body Mass Index</strong></td>
<td>29.04 ± 5.25</td>
<td>27.43 ± 5.05</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>ADCS-ADL</strong></td>
<td>55.79 ± 15.02</td>
<td>56.67 ± 15.63</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>ADAS-Cog</strong></td>
<td>22.88 ± 10.51</td>
<td>26.28 ± 17.35</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>MMSE</strong></td>
<td>19.45 ± 4.21</td>
<td>17.95 ± 5.66</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>CDR</strong></td>
<td>0.82 ± 0.45</td>
<td>1.02 ± 0.6</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Presented as Mean ± Standard deviation, except gender and ethnicity.

MMSE: Mini Mental State Examination; ADAS-cog: Alzheimer’s Disease Assessment Scale-cognitive subscale; ADCS-AD: Alzheimer’s Disease Cooperative Study - Activities of Daily Living; CDR: Clinical Dementia Rating Scale
Table 2

List of adverse effects

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Treatment</th>
<th>Adverse Event</th>
<th>SOC</th>
<th>AE Intensity</th>
<th>Relationship to IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR10006</td>
<td>Active</td>
<td>Pruritus</td>
<td>Skin and subcutaneous tissue disorders</td>
<td>Mild</td>
<td>Unrelated</td>
</tr>
<tr>
<td>TR10011</td>
<td>Active</td>
<td>Dizziness</td>
<td>Nervous system disorders</td>
<td>Mild</td>
<td>Unrelated</td>
</tr>
<tr>
<td>TR10034</td>
<td>Active</td>
<td>Diarrhea</td>
<td>Gastrointestinal disorders</td>
<td>Moderate</td>
<td>Unrelated</td>
</tr>
<tr>
<td>TR10036</td>
<td>Active</td>
<td>Diarrhea</td>
<td>Gastrointestinal disorders</td>
<td>Moderate</td>
<td>Unrelated</td>
</tr>
<tr>
<td>TR20045</td>
<td>Active</td>
<td>Nausea</td>
<td>Gastrointestinal disorders</td>
<td>Moderate</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

We measured clinical variables in all patients and analysed the differences before and after administration in the active and placebo groups (Fig. 1B, Table 3, Dataset S2). As decreased ADAS-Cog score is the indicator of the improved cognitive function in AD patients, the ADAS-Cog scores is significantly decreased on Day 28 vs Day 0 (Log2FoldChange (FC) = -0.33, (26% improvement), p-value = 0.0000003, effect size = -0.43, 95% CI = [-0.59, -0.29]) and further decreased on Day 84 vs Day 0 (Log2FC= -0.37, (29% improvement), p-value = 0.00001, effect size= -0.50, 95% CI= [-0.71, -0.29]) in the CMA group. A slightly but significant improvement is also found in the placebo group on Day 28 vs Day 0 (Log2FC= -0.16, (12% improvement), p-value = 0.009, effect size= -0.16, 95% CI= [-0.27, -0.05]) and Day 84 vs Day 0 (Log2FC= -0.19, (14% improvement), p-value = 0.001, effect size= -0.23, 95% CI= [-0.35, -0.11]). There was no significance between groups on Day 28 and Day 84. This could be related to the placebo effect which is apparent in the early stages of AD clinical trials showing relatively unchanged response or even improvement that may continue for up to 12 months, followed by natural disease progression overtime when the drug effect becomes more prominent and the placebo response decreases (40).

Table 3 Differences in ADAS-Cog, ADCS-ADL and MMSE scores in the CMA and placebo groups

<table>
<thead>
<tr>
<th>Measurements</th>
<th>PLACERO</th>
<th>CMA</th>
<th>CMA vs PLACERO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log2FoldChange</td>
<td>P value</td>
<td>Log2FoldChange</td>
</tr>
<tr>
<td>Day 28 vs 0</td>
<td>Day 84 vs 0</td>
<td>Day 28 vs 0</td>
<td>Day 84 vs 0</td>
</tr>
<tr>
<td>ADAS-COG</td>
<td>-0.089</td>
<td>-0.069</td>
<td>-0.089</td>
</tr>
<tr>
<td>High score</td>
<td>0.080</td>
<td>0.055</td>
<td>0.080</td>
</tr>
<tr>
<td>Low score</td>
<td>-0.031</td>
<td>-0.023</td>
<td>-0.031</td>
</tr>
<tr>
<td>MRI</td>
<td>0.010</td>
<td>0.009</td>
<td>0.010</td>
</tr>
<tr>
<td>ADCS-ADL</td>
<td>-0.024</td>
<td>-0.024</td>
<td>-0.024</td>
</tr>
<tr>
<td>All</td>
<td>0.078</td>
<td>0.078</td>
<td>0.078</td>
</tr>
<tr>
<td>Mini Mental</td>
<td>-0.043</td>
<td>0.022</td>
<td>-0.043</td>
</tr>
<tr>
<td>All</td>
<td>0.087</td>
<td>0.064</td>
<td>0.087</td>
</tr>
</tbody>
</table>

MMSE: Mini Mental State Examination; ADAS-cog: Alzheimer’s Disease Assessment Scale-cognitive subscale; ADCS-AD: Alzheimer’s Disease Cooperative Study - Activities of Daily Living; ADAS-Cog score > 20 is high, ≤20 is low; MRI: Magnetic resonance imaging
We also analysed the differences between clinical parameters by stratifying the patients into low-scored and high-scored ADAS-Cog groups (> 20 ADAS-Cog score is high, n = 30; ≤20 score is low, n = 39). There was a significance (Log2FC= -0.77, p-value = 0.0073, effect size= -1.23, 95% CI= [-0.3,-2.17]) between CMA and placebo groups in patients with higher ADAS-Cog scores on Day 84 (Fig. 1B, Dataset S2). Moreover, we found a significant improvement of ADAS-Cog scores between Day 28 vs Day 0 (Log2FC= -0.31, (24% improvement), p-value = 0.002, effect size= -0.48, 95% CI= [-0.76, -0.2]) and Day 84 vs Day 0 (Log2FC= -0.38, (30% improvement), p-value = 0.003, effect size= -0.59, 95% CI= [-0.97, -0.21]) in the severe CMA group and no significance difference in the severe placebo (p > 0.05 in both time points) group (Fig. 1B, Dataset S2). As shown in Fig. 1B, we observed a significant difference in the baseline value distribution and mean of ADAS-Cog scores in the severe (ADAS-Cog > 20) CMA and placebo groups due to the randomisation of the subjects. To verify our results, we selected 10 patients from the CMA group with matched ADAS-Cog values to the placebo group (p-value: 0.693) and presented the ADAS-Cog scores in Fig. 1C. We recalculated the differences in ADAS-Cog scores and again found significant improvement in the CMA group, whereas we found no significant difference in the placebo group. Our results indicated that the severe AD patients with high ADAS-Cog scores are more responsive to CMA.

Other primary endpoints were Alzheimer’s Disease Cooperative Study - Activities of Daily Living (ADCS-ADL) and Mini Mental State Examination (MMSE). No significance was found between time points and between groups in ADCS-ADL (Fig. 1B, Table 3, Dataset S2). For MMSE, a significance in the placebo was seen when comparing Day 28 and Day 84 to Day 0 (p-value = 0.04 and p-value = 0.02, respectively) as well as in CMA group when comparing Day 28 to Day 0 (p-value = 0.02) but not in Day 84 to Day 0 (Fig. 1B, Table 3, Dataset S2). There was no significance in ADCS-ADL and MMSE in any time points and between groups in patients evaluated with MRI (Dataset S2).

Analysis of secondary outcome variables showed that serum alanine aminotransferase (ALT) levels (Log2FC= -0.38, p-value = 0.01) and the uric acid levels (Log2FC= -0.19, p-value = 0.001) were significantly lower on Day 84 vs Day 0 only in the CMA group (Fig. 1D, Dataset S2). This reduction was seen both in high- and low-ALT level groups. In contrast, we found no significantly altered parameters on Day 84 vs Day 0 in the placebo group (Fig. 1D, Dataset S2).

We also measured the complete blood count parameters and found that their levels were significantly changed in the CMA group (Fig. 1D, Dataset S2). We found that the levels of platelets, basophil % and absolute numbers of basophil and neutrophil were significantly lower on Day 84 vs Day 0 only in the CMA group. In contrast, we found that the levels of monocytes were significantly increased on Day 84 vs Day 0 in the CMA group (Fig. 1D, Dataset S2). Hence, our analysis indicated that the administration of CMA improved the clinical parameters in parallel to the improvement in cognitive functions in AD patients.

Blood profile informs the response to CMA

Treatment response variability and clinical heterogeneity in AD are well documented in the literature. We observed interindividual variability in clinical measures in responses to CMA administration. Therefore,
we hypothesised that some patients would respond better to CMA than others and that clinical measurements could define these subsets.

To determine whether alanine transferase (ALT), a marker for liver damage, could indicate a better response to CMA, we stratified the patients into high and low ALT groups by the median ALT of all patients on Day 0. As shown in Fig. 2A, the patients of the CMA group with low ALT achieved a significant improvement in ADAS-Cog score over different time points, while the patients in the placebo group had no improvement. In contrast, the patients of the CMA group with high ALT levels also exhibited an improved (i.e., decreased) ADAS-Cog score, but the degree of change was not as much as the patients in the CMA group with low ALT levels. Moreover, patients in the placebo group with high ALT levels also had improved ADAS-Cog scores. Thus, these results suggest that the patients with low ALT levels are more responsive to CMA.

We repeated this stratification for each blood parameter to determine the patient conditions in which CMA produces a tremendous response (Fig. 2B). In addition to low ALT, we identified high alkaline phosphatase (ALP), low gamma-glutamyl transferase (GGT), high hematocrit, high HbA1c, high insulin, high uric acid, high basophil count, and high red blood cell count as indicators for better responsiveness to CMA.

**CMA Increases the Plasma Levels of Metabolites Associated with Metabolic Activators**

We first analysed the plasma levels of serine, carnitine, NR, cysteine and their by-products. CMA administration increased the plasma levels of metabolic activators on Day 84 vs Day 0 in the CMA group (Fig. 3A, Dataset S3). Moreover, the plasma levels of NR, 1-methylnicotinamide, nicotinurate, N1-methyl-2-pyridone-5-carboxamide and nicotinamide (associated with NR and NAD + metabolism); of serine, glycine and sarcosine (associated with serine and glycine metabolism); and of deoxycarnitine and carnitine (associated with carnitine metabolism) were significantly higher in the CMA group on Day 84 compared to Day 0.

Next, we investigated the relationship between the plasma level of administrated metabolic activators and other metabolites. We analysed 195 of the most significantly correlated plasma metabolites with serine, L-carnitine, NR, and cysteine (Dataset S4). We found two clusters of metabolites that are significantly correlated with cysteine only or together with serine, carnitine and NR. We observed that cysteine had different plasma changes than the other three metabolic activators, as reported in previous clinical trials (29, 41).

**Effect of CMA on Global Metabolism**

We identified the significantly (FDR < 0.05) different plasma metabolites on Day 84 vs Day 0 and found that the plasma levels of 132 metabolites were significantly different in the CMA group (Fig. 3, Dataset S3). Evaluation of plasma metabolites that differed significantly on Day 84 vs Day 0 in each group showed that a larger number of metabolites related to amino acid (n = 53), lipid metabolism (n = 42) and
other metabolic pathways (n = 37) were altered in the CMA group compared to the placebo group (Fig. 3, Dataset S3).

N-acetyl aspartate (NAA) is one of the most abundant brain metabolites, and its reduced plasma levels are associated with brain tissue damage. Previous research revealed the importance of NAA in maintaining energy metabolism in the central nervous system (42). We observed that plasma levels of NAA significantly increased on Day 84 vs Day 0 in the CMA group (Fig. 3B, Dataset S3). Another upregulated metabolite on Day 84 vs Day 0 in the CMA group is sarcosine (a derivative of glycine) which has been widely studied for its improving effects on cognitive symptoms by different pharmacological activities in neurons (43). Of note, quinolinic acid (an endogenous excitotoxin acting on N-Methyl-D-aspartate receptors leading to neurotoxic damage) levels significantly decreased on Day 84 vs Day 0 only in the CMA group (Fig. 3B, Dataset S3).

Increased plasma homocysteine levels are a known risk factor for AD, and several animal studies implicated the promising results of methionine restriction (44, 45). In our clinical trial, plasma levels of S-adenosylhomocysteine, 2,3-dihydroxy-5-methylthio-4-pentenoate (DMTPA) and N-acetyl taurine were significantly downregulated on Day 84 vs Day 0 in the CMA group (Fig. 3B, Dataset S3). Of note, reductions of these metabolites are significantly correlated with serine and NR supplementation (Fig. 4A, Dataset S4).

Increased plasma levels of metabolites in the kynurenine pathway are associated with AD severity (44). In our study, we found that plasma levels of kynurenate and 8-methoxykynurenate were significantly lower on Day 84 vs Day 0 in the CMA group (Fig. 3B, Dataset S3). Reduction in the plasma level of kynurenate was positively correlated with plasma serine levels (Dataset S4). Kynurenate, which has a prooxidant effect, is the product of the tryptophan degradation pathway. Its aerobic irradiation produces superoxide radicals and leads to cytochrome C reduction (46). It has been reported that increased levels of kynurenine lead to cell death through the reactive oxygen species (ROS) pathway in nature killer (NK) cells (47) and lower blood pressure in systemic inflammation (48).

Emerging evidence indicates a link between abnormal kidney function and AD, but the potential impact of the kidney on cognitive impairment is still undetermined (49). Recent studies showed that plasma levels of N,N,N-trimethyl-5-aminovalerate are involved in lysine metabolism, and it serves as an indicator of elevated urinary albumin excretion (50). Here, we found that the plasma level of N,N,N-trimethyl-5-aminovalerate was significantly decreased on Day 84 vs Day 0 in the CMA group (Fig. 3B, Dataset S3) and significantly inversely correlated with the plasma level of serine and NR. Moreover, the plasma level of creatinine was also significantly decreased on Day 84 vs Day 0 in the CMA group (Fig. 3B, Dataset S3). The plasma reduction in creatinine is inversely correlated with the plasma level of serine (Dataset S4). Additionally, our analysis revealed decreased levels of several metabolites belonging to histidine metabolism in the CMA group on Day 84 vs Day 0. Among those N-acetyl-1-methylhistidine is associated with chronic kidney disease and showed a significant negative correlation with serine supplementation (Fig. 3B, Dataset S4). Also, we found that plasma levels of metabolites related to the urea cycle (3-amino-
2-piperidone, arginine, homoarginine, N-alpha-acetylornithine, ornithine and pro-hydroxy-pro) were significantly decreased in the CMA group on Day 84 vs Day 0 (Fig. 3B, Dataset S3) and inversely correlated with the plasma level of serine and NR (Dataset S4).

Lipids play a fundamental role in the pathophysiology of neurodegenerative diseases, including AD. Specific lipid species of cellular membranes (e.g., cholesterol and sphingolipids) are structural components of cell membranes and regulate many critical aspects of brain functions(51). In our study, plasma levels of many metabolites associated with sphingomyelins and fatty acid metabolism (acyl carnitines) were significantly increased on Day 84 vs Day 0 in the CMA group (Fig. 3C, Dataset S3). Interestingly, plasma levels of pregnenolone steroids and 2R,3R-dihydroxybutyrate were significantly decreased on Day 84 vs Day 0 (Fig. 3C, Dataset S3). These alterations were significantly positively correlated with carnitine and serine levels (Dataset S4).

Effect of CMA on Plasma Proteins

Plasma levels of 1466 protein markers were measured with the plasma proteome profiling platform Proximity Extension Assay quantifying the plasma level of target proteins. After quality control and exclusion of proteins with missing values in more than 50% of samples, 1463 proteins were analysed (Dataset S5). Proteins whose levels differed significantly between the visits in the CMA and placebo groups are listed in Dataset S5.

We analysed the effect of CMA on plasma protein profile and found that 22 proteins were significantly (p-value < 0.01) different in the CMA group on Day 84 vs Day 0. Nineteen of these proteins were significantly decreased, whereas 3 of these proteins were significantly increased on Day 84 vs Day 0. After filtering out the proteins based on log2FC, we found that the plasma levels of PSPN, OSM, PADI4, PDGFC, SCGN, LTBP3, CLEC4G, MERTK, WNT9A, ISM1, ASAH2, CES3, HPGDS, NPY, THPO, SIGLEC6, GDNF, PADI2 and EGFL7 were significantly downregulated in the CMA group. The plasma level of KLB, BGN, and ST3GAL1 was significantly upregulated in the CMA group (Fig. 4B, Dataset S5). We observed that only one significantly (p-value < 0.01) altered protein - EGFL7 upregulated- in the placebo group (Fig. 4B, Dataset S5).

The proteomic analysis in this study revealed significant alteration in levels of several critical proteins that play an essential role in the pathogenesis of AD. For instance, levels of MertK (52, 53), EGFR (54, 55), oncostatin (56–60), PAD4 (61, 62), LTGF (63–67), and TPO (68), known as a potent inducer of neuroinflammation, amyloid production and apoptosis, decreased. In contrast, proteins with neuroprotective and pro-cognitive properties, such as Klotho (69, 70) and ST3GAL1 (71), increased after CMA treatment. More interestingly, most of the analysed proteins were also significantly altered in recent human AD studies (63–67, 72–78). KlothoB levels were also significantly altered after CMA treatment, consistent with their neuroprotective role as a cofactor and neurotrophic factor. Recent studies have shown that KlothoB indirectly regulates glucose and energy metabolism through F2F1, expressed in some regions of the brain involved in learning and memory (77). Moreover, GABA signalling has also been shown to play a critical role in mediating the detrimental effects of increased dihydroxybutyrate levels in the progression
of MCI (79). Interestingly, our metabolomic study indicated decreased post-therapeutic dihydroxybutyrate levels. Although the exact pathways involved in the metabolic generation of DHBA are still far from clear, it has been hypothesised that dihydroxybutyrate levels may be a compensatory response to increased cellular stress secondary to compromise of the Krebs cycle function, creating an alternative energy production pathway in AD (79). This represents indirect evidence to suggest that our treatment exhibits an energetic regulatory function.

**Integrative Multi-Omics Analysis**

Multi-omics data integrations have been proven to give novel insights and a more holistic view of the human body in both health and disease states (80). In this study, we generated an integrative multi-omics network using metabolomics and proteomics data, coupled with detailed clinical variables, to understand the functional relationships between analytes from the same and different omics data types. We generated the network using the method used in iNetModels (39), to which we also deposited our network. The network consists of 937,282 edges from 2,273 nodes (36.3% network density, Dataset S6).

We extracted a sub-network to highlight the interactions between the individual metabolic activators, cognitive function (ADAS-Cog scores), two highlighted proteins (OSM and PSPN), and their top neighbours (Fig. 4C). From the sub-network, ADAS-Cog was negatively associated with carnitine (and its derivatives) and nicotinamide associated metabolites, whereas the metabolic activators were negatively associated with fatty acid and histidine metabolism. Finally, we observed that, among others, OSM and PSPN were positively associated with immune and cell cycle-related proteins.

Subsequently, we performed centrality analysis to identify the most central analytes in the networks. The top 20 most central metabolites were dominated by amino acid metabolites (tryptophan, glutamate, and branched-chain amino acid metabolism) and lipid metabolites (androgenic steroid pathway), where top proteins were related to, among others, short- and long-term memory (CALB1), lipid metabolism (PLA2G10), and immune response (SELPLG, CLEC4D, and LGALS7).

Furthermore, we performed community analysis within the network using the Leiden algorithm. We discovered 3 modules that showed significant interaction among the members. In cluster-0, the biggest clusters, the top nodes were related to tryptophan metabolism (indole acetate), fatty acid metabolism (3hydroxyoctanoate), and steroid metabolism (11-ketoetiocholanolone glucuronide and 11-beta-hydroxyetiocholanolone glucuronide). Moreover, we found 2 top proteins in the same cluster, ACTA2 and IGFBP1, associated with AD (81, 82). In cluster-1, the top nodes were associated with leucine metabolism (3-hydroxy-2-ethylpropionate), ceramide phosphatidylethanolamine, and a carnitine metabolite (erucylcarnitine); meanwhile, cluster-2’s central nodes were related to methionine metabolism and aminosugar metabolism (N-acetylgluosamine/N-acetylgalactosamine). These results showed that the integrative multi-omics network analysis could be used to strengthen the results from single omics analyses and identify key analytes associated with AD. Moreover, it provided new insights by elucidating the functional relationships within and between different omics data.
In evaluating the correlations between each activators (used in the present study for therapeutic purposes) and clinical, metabolic, and proteomic parameters, we identified significant correlations between serine, carnitine, cysteine, and nicotinamide levels and improved peripheral blood parameters, such as liver function, CBC, and glycated hemoglobin (HbA1c), which are relevant to the pathogenesis of AD. Accordingly, improved ADAS-Cog scores were also associated with serum serine and carnitine changes, which fit well with their well-known pro-cognitive and energy-boosting effects. Similar results were also observed for metabolomic and proteomic data. The majority of the activators exhibited significant correlations with improved metabolites and proteins (either increased or decreased) relative to a slower degeneration process in AD. It is worth mentioning here that two of the proteins, OSM and PSPN, most strongly associated with other beneficial protein metabolites, were also related to several critical amino acid alterations, such as spermidine and hypotaurine, which may suggest a metabolic shift from the protein to amino acid metabolism to compensate the energy deficit reported in AD.

**Effect of CMA on Hippocampal volumes and Cortical Thickness**

The MRI group's baseline demographics and improved clinical parameters significantly aligned with the entire patient cohort (Dataset S1&S2). Based on comparing the differences between pre and post-treatment groups in each treatment arm (83), our results showed that the left whole hippocampal mean volume, and left molecular layer HP body were significantly improved after the CMA treatment (p < 0.05, Fig. 5B, Dataset S7). Several other sub-anatomic hippocampal regions (left CA1 body, left whole hippocampal body) were improved near to a statistically significant level (p < 0.05, Fig. 5B, Dataset S7) after the CMA treatment. According to longitudinal cortical thickness analysis, the active group showed statistically significant alterations in the bilaterally lateral occipital, bilateral rostral middle frontal, left inferior parietal, and left paracentral regions (Fig. 5C).

**Discussion**

We showed that oral administration of CMA has a profound effect on cognitive function after 84 days of treatment in AD patients based on ADAS-Cog scores. The cognitive functions in high scored patients are improved while there were no differences in the placebo group. We also showed beneficial effects both in severe and mild patients that cognitive functions in both AD patients are improved 29% in the CMA group, whereas only 14% in the placebo group after 84 days, consistent with a placebo effect that is seen in the early stages of AD clinical trials (40, 84, 85). Improvement in cognitive functions was supported by positive alterations on hippocampal subfield volumes and cortical thickness in the CMA group, while no significant changes were found in the placebo group. Our finding of beneficial effects in severe AD is of particular value since severe AD patients lack current therapeutic regimes, except for palliative support. Apart from clinical severity, we observed that various clinical variables were also related to the treatment response. For example, patients with low ALT and showed an increased metabolic load (i.e., increased HbA1c and insulin levels) or impaired CBC values responded better to treatment.
The effect of oral administration of CMA was substantiated with a comprehensive analysis of protein and metabolites in the plasma of the patients using a multi-omics analytical platform. The clinical results are consistent with the genome-scale metabolic modelling of more than 600 AD patients showing clear evidence of mitochondrial dysfunction. It is also consistent with the results from an animal model demonstrating improved AD-associated histological parameters in animals treated with oral administration of CMA (under review). Thus, the present study suggests an attractive therapeutic regime for improving mitochondrial dysfunction in AD patients.

Considering the role of hippocampal and frontoparietal degeneration in AD pathogenesis, our neuroimaging observation of improved hippocampal subfield volumes and cortical thickness after metabolic stimulation was unsurprising. Herein, we observed significantly improved hippocampal volumes and frontal and parietal cortical thickness associated with improved cognitive functions in the CMA patients, while no beneficial effects were observed in the placebo group, suggesting a real drug effect in the CMA group reflected in major cognitive brain regions in AD. Our findings of improved hippocampal volumes and specific cortical thicknesses are also in line with the data showing that the hippocampus and other AD-relevant cortical regions are mostly glucose-dependent and most of the brain’s early and energy-dependent degenerating regions (86–88) which might make them especially responsive to metabolic interventions. Shortly our neuroimaging results might indicate that the administration of CMA affected the global metabolism of AD patients and positively affected AD-relevant energetic structures.

The metabolomics data confirmed the expected biological outcomes of CMA treatment. Levels of plasma nicotinamide and related metabolites increased, suggesting that NR provided sufficient substrate for mitochondrial fatty acid oxidation. In addition to its role as a cellular metabolite, NAD + functions as an essential cofactor for the DNA repair protein PARP1 (17). Hyperactivation of PARP1 and decreased NAD + have been already identified in the brains of patients with AD (89, 90). Serine plasma levels also increased, suggesting that CMA treatment improves the serine deficiency associated with AD. For instance, a recent study showed that the adenosine triphosphate (ATP)-reducing the effect of glucose hypometabolism was restored with oral serine supplementation, suggesting the potential use of oral serine as a ready-to-use therapy for AD (91). The exact mechanism of action also applies to cysteine. As a glutathione precursor, cysteine acts as an antioxidant and anti-inflammatory agent, maintaining the mitochondrial homeostasis and key neurotransmitter systems, such as glutamate, involved in learning and memory (92, 93). Accordingly, NAC has been tested as a medication in AD and exhibited effects suggestive of future potential use as an alternative medication (94). More importantly, fatty acid oxidation and carnitine metabolism were significantly facilitated, as shown by the robust increase in plasma levels of carnitine. These findings fit well with recent human data showing that severe disturbances in carnitine metabolism frequently occur in individuals with AD, in association with severe mitochondrial dysfunction (95, 96). Cristofano et al. showed a progressive decrease in carnitine serum levels in individuals shifting from normal status to AD, suggesting that decreased serum concentrations of carnitine may predispose them to AD (97). In support of this hypothesis, human clinical studies have demonstrated the pro-cognitive effects of carnitine in mild cognitive impairment (MCI) and AD(98–100).
This, in turn, led to the suggestion that stabilising the bioenergetic balance may slow or even reverse MCI and the progression of dementia in patients with AD.

In addition, the levels of tryptophan metabolites, including kynurenic acid, kynurenic acid, and tryptophan betaine, decreased significantly after CMA treatment. Increased levels of these metabolites were previously associated with increased neurodegeneration and clinical cognitive impairment through a high oxidative load and the formation of neurofibrillary tangles (NFTs) (101, 102). For instance, recent data showed a synergistic relationship between β-amyloid 1–42 and enzymatic activations of the tryptophan kynurenine pathway, resulting in increased oxidative stress, which may be associated with the formation of NFTs and senile plaque development (103). Also, one recent study revealed that tryptophan-2,3-dioxygenase (TDO) was highly expressed in the brains of patients with AD and co-localised with quinolinic acid, NFTs, and amyloid deposits in the hippocampus of post-mortem brains of patients with AD (104).

We also observed significantly increased levels of NAA, sarcosine, methionine, cysteine, and S-adenosylmethionine (SAM) and decreased levels of histidine, tryptophan quinolate, and urea cycle metabolites, which play a critical role in cognitive and mitochondrial functions. For instance, increased NAA may provide an additional energy source for intercellular metabolite trafficking during the neurodegenerative process, especially when glucose metabolism is downregulated (42). Similarly, increased sarcosine levels may boost cognition, as previously shown in patients with schizophrenia, in which oxidative damage and impaired glucose metabolism play key roles (105). In addition, decreased histidine metabolism and other decreased markers, such as homocysteine and S-adenosylhomocysteine (SAH) found in our treatment group, have been already shown to slow the cognitive ageing process appropriately downregulated (106). For instance, increased plasma homocysteine levels are a known risk factor for AD, whereas a low leucine and arginine diet yields beneficial cognitive effects (107).

Interestingly, CMA rapidly lowered uric acid and associated metabolites levels. Uric acid stimulates inflammation either directly or by activating NLRP3 inflammasomes (108). Although the extent to which uric acid reduction contributed to the regression in cognitive impairment is unclear, it is likely linked to improved metabolic homeostasis. A good example is a recent clinical study showing increased urea metabolism in patients with AD (109). Accordingly, decreased taurine levels and urea metabolites are associated with a diminished risk of dementia (110). The majority of clinical study findings collectively agree with our results, showing significantly dysregulated baseline metabolites normalised with treatment.

To date, a few studies aimed to identify global changes in metabolites and metabolic pathways in AD (15, 111, 112). Among these, some studies highlight that lipid dysfunction also plays an essential role in the pathophysiology of AD (113). In terms of lipid metabolism, significant differences in the levels of some compounds have been observed in patients with AD. Despite some discrepant trends in cross-sectional studies examining lipids in AD patients (114, 115), the plasma levels of sphingolipids, sphingomyelins (116, 117), acylcarnitines (118) and phosphatidylcholines (PC) (119–121) exhibited
statistically lower concentrations in patients with AD, even in the preclinical stages of the disease (18). In addition, a significant correlation among different lipid metabolites, tau and amyloid pathology, brain atrophy and cognitive decline was observed in an AD human study (18). An autopsy study of frontal cortex metabolites from patients with AD showed that impaired glycerophospholipid metabolism was involved in six central metabolic pathways altered in the disease (122). In brief, we observed significantly increased post-therapeutic levels of lipid metabolites, previously reported to decrease in patients with AD, including sphingomyelin, carnitine and carnitine-related by-products.

Despite insufficient clinical AD data concerning cholesterol metabolites and dicarboxylic acids (DCAs), we observed significantly lower levels of these metabolites after CMA treatment (123). Levels of pregnanediol, a metabolite of pregnenolone, and DCAs, end-products of β- or omega oxidation, which were observed as decreased in the present study, were previously reported to be lower in the urine of patients with AD (124, 125). Considering the neurotoxic role of bile acids, along with the oxidative properties of DCAs, the detection of decreased levels of bile acid metabolites and DCA products in the present study is therefore not surprising. Similarly, allopregnanolone has already been reported to have harmful effects on cognitive functions through gamma-aminobutyric acid (GABA) signalling (126). Also, increased bile acid levels have been reported in MCI and AD. In contrast, bile acids strongly inhibited the cysteine catabolic pathway in the preclinical period, resulting in depletion of the free cysteine pool and reduced antioxidant glutathione concentrations (127).

The study has limitations to be considered. One feasible limitation of the study is the small sample size after classifying the patients into low- and high-scored ADAS-Cog groups. Therefore, a clinical trial with a larger sample size would be more beneficial to elucidate the effects of CMA on functional and structural brain alterations. Another limitation is that no ApoE genotyping is performed, that would be informative for the risk variants for AD patients.

**Conclusions**

Based on the human phase 2 clinical study, we observed that oral administration of CMA improves mitochondrial dysfunction in AD patients. The safety profile of metabolic activators in these patients was consistent with the results of our previous one-day calibration study and clinical phase 2 and phase 3 trials, including only a single component of the CMA (25). Our present study showed that CMA was safe and well-tolerated, and no major safety concerns were identified. Importantly, CMA improved cognition and serum markers, especially in patients with severe AD after only 12 weeks of treatment, suggesting further studies with severe AD patients. Considering all these findings, our data provide strong evidence that targeting multiple pathways by metabolic activators is a potentially effective therapeutic strategy for AD. Furthermore, we were also able to correlate metabolite levels and structural imaging data with typical measures of cognition, very likely a unique finding bridging system-level biology to specific cognitive regions in the AD brain.

**Abbreviations**
AD
Alzheimer's disease
ADAS-Cog
Alzheimer’s disease Assessment Scale-cognitive subscale
ADCS-ADL
Alzheimer's Disease Cooperative Study - Activities of Daily Living
ALT
Alanine transferase
CDR-SOB
Clinical Dementia Rating Scale Sum of Boxes
CMA
Combined Metabolic Activators
DMTPA
2,3-dihydroxy-5-methylthio-4-pentenoate
GGT
gamma-glutamyl transferase
HbA1c
Glycated hemoglobin
MMSE
Mini Mental State Examination
NK
Nature killer cells
NR
Nicotinamide riboside
ROS
Reactive oxygen species

Declarations

Ethical approval and consent to participate

Written informed consent was obtained from all participants before initiating any trial-related procedures. The trial was conducted following Good Clinical Practice guidelines and the principles of the Declaration of Helsinki. The ethics committee approved the study of Istanbul Medipol University, Istanbul, Turkey (Date:22.01.2020, Decision No: 7), and registered at https://clinicaltrials.gov/ with Clinical Trial ID: NCT04044131.

Consent for publication

Written informed consent for publication was obtained from all participants before initiating any trial-related procedures.
**Availability of supporting data**

The data supporting the findings of this study are available in Supplementary Material. Raw data are available from the corresponding author, upon reasonable request.

**Competing interests**

AM, JB and MU are the founder and shareholders of ScandiBio Therapeutics. The other authors declare no competing interests.

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**Author contributions**


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**References**


**Figures**
CMA Improves ADAS-Cog scores and clinical parameters.

A) Study design for testing the effects of CMA in AD patients. B) Differences in ADAS-Cog scores in the CMA and placebo groups on Days 0, 28 and 84 are presented. Additionally, differences in ADAS-Cog scores were analysed by stratifying the patients into high and low levels of ADAS-Cog groups (> 20 ADAS-
Cog is high, ≤ 20 is low). As decreased ADAS-Cog score is the indicator of the improved cognitive function in AD patients, the ADAS-Cog scores is significantly decreased on Day 28 vs Day 0 (Log2FoldChange (FC)= -0.33, (26% improvement), p-value=0.0000003) and Day 84 vs Day 0 (Log2FC= -0.37, (29% improvement), p-value=0.00001) in the CMA group. A slightly but significant decrease is found in the placebo group on Day 28 vs Day 0 (Log2FC= -0.16, (12% improvement), p-value=0.009) and Day 84 vs Day 0 (Log2FC= -0.19, (14% improvement), p-value=0.001). The differences between clinical parameters have also been analysed by stratifying the patients into low-scored (mild patients) and high-scored (severe patients) ADAS-Cog groups (>20ADAS-Cog score is high, ≤20 is low). The ADAS-Cog scores is significantly decreased on Day 28 vs Day 0 (Log2FC= -0.31, (24% improvement), p-value=0.002) and Day 84 vs Day 0 (Log2FC=-0.38, (30% improvement), p-value=0.003) in the high scored CMA group and no significance difference in the high-scored placebo (p>0.05 in both time points) group. C) We selected 10 patients from the severe (ADAS-COG > 20) CMA group with matched ADAS-COG values to the placebo group (P-Value: 0.693) and presented the ADAS-Cog scores. We recalculated the differences in ADAS-COG scores and found significant improvement in the CMA group whereas there was no significant difference in the placebo group. D) Heatmaps show log2FC based alterations of the clinical variables, compared before and after the administration of CMA in both drug and placebo groups. Asterisks indicate statistical significance based on Student's t-test. P-value <0.05. Log2FC: log2(fold change).
Figure 2

Identification of clinical variables informative for response to CMA administration.

A) Distribution of ADAS-Cog scores over visit number for patients with ALT ≤ 16 IU/L at visit 1 (upper panel) and patients with ALT > 16 IU/L at visit 1 (lower panel). B) Between-visit changes to ADAS-Cog with various clinical variable groupings are shown. Only those clinical variable groupings resulted in a
more significant change to ADAS-Cog in the CMA group compared to the placebo group (a p-value of 0.05 or better is shown). The Colour scale indicates log2 fold change to ADAS-Cog between visits. Statistical significance between visits was determined by a paired t-test across individuals who attended both visits. Asterisks indicate a statistical significance of p< 0.05.

Figure 3
CMA alters plasma metabolite levels

A) Differences in the plasma levels of individual CMA, including serine, carnitine, cysteine and nicotinamide, are shown in the CMA and placebo groups on Days 0 and 84. Plasma levels of B) amino acids, C) lipids and D) other metabolites that are significantly different between Day 84 vs Day 0 in the CMA and placebo groups are presented. Adj. p< 0.05. Heatmap shows log2FC values of metabolites between Day 84 vs Day 0. Asterisks indicate statistical significance based on paired Student’s t test. Adj.p< 0.05. Log2FC: log2(fold change).
Figure 4

Correlation of CMA with plasma metabolites and altered plasma protein levels

Associations between the plasma level of individual CMA and the 10 most significantly correlated plasma metabolites are presented. Asterisks indicate statistical significance (Adj.p < 0.05) based on Spearman correlation analysis. Cor.Coeff: Correlation coefficient B) Heatmap shows log2FC based
alterations between the significantly different proteins on Day 84 vs Day 0 in the CMA and placebo groups. Asterisks indicate statistical significance based on paired Student’s t-test. p < 0.01. C) Integrated multi-omics data based on network analysis represents the neighbours of the CMA, including serine, carnitine, nicotinamide and cysteine, and ADAS-Cog scores. Only analytes that are significantly altered in CMA Day 84 vs Day 0 are highlighted.

Figure 5

**Structural magnetic resonance imaging analysis**

A) Increased cortical thickness (red-yellow) in the study group (p<0.05) in the inferior parietal, lateral occipital and middle frontal and paracentral cortical regions based on the illustration of FreeSurfer’s Qdec application. B) Segmentation maps of the hippocampal subfields displayed on the axial, sagittal and coronal planes. C) Hippocampal subfield measurements showing the increased volumes in the study group (p<0.05) in the i) left mean hippocampal and ii) left hippocampal molecular layer based on the FreeSurfer’s Qdec application. Also, several other sub-anatomic hippocampal regions iii) left CA1 body and iv) left whole hippocampal body were improved near to a statistically significant level (p<0.05)

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- YulugEtalADFigureS1.png
- YulugEtALADsupplementaryAppendix.docx
- YulugEtalADDatasetS1.xlsx
- YulugEtalADDatasetS2.xlsx
- YulugEtalADDatasetS3.xlsx
- YulugEtalADDatasetS4.xlsx
- YulugEtalADDatasetS5.xlsx
- YulugEtalADDatasetS6.txt
- YulugEtalADDatasetS7.xlsx
- YulugADCONSORTv2TNEU.docx