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Longitudinal metabolomics analysis reveals the acute effect of cysteine and NAC included in the combined metabolic activators

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ABSTRACT

Growing evidence suggests that the depletion of plasma NAD^+ and glutathione (GSH) may play an important role in the development of metabolic disorders. The administration of Combined Metabolic Activators (CMA), consisting of GSH and NAD^+ precursors, has been explored as a promising therapeutic strategy to target multiple altered pathways associated with the pathogenesis of the diseases. Although studies have examined the therapeutic effect of CMA that contains N-acetyl-L-cysteine (NAC) as a metabolic activator, a system-wide comparison of the metabolic response to the administration of CMA with NAC and cysteine remains lacking. In this placebo-controlled study, we studied the acute effect of the CMA administration with different metabolic activators, including NAC or cysteine with/without nicotinamide or flush free niacin, and performed longitudinal untargeted-metabolomics profiling of plasma obtained from 70 well-characterized healthy volunteers. The time-series metabolomics data revealed the metabolic pathways affected after the administration of CMAs showed high similarity between CMA containing nicotinamide and NAC or cysteine as metabolic activators. Our analysis also showed that CMA with cysteine is well-tolerated and safe in healthy individuals throughout the study. Last, our study systematically provided insights into a complex and dynamics landscape involved in amino acid, lipid and nicotinamide metabolism, reflecting the metabolic responses to CMA administration containing different metabolic activators.

1. Introduction

Metabolic disorders have become a growing worldwide health burden due to their dramatically increasing prevalence [1]. Accumulating evidence implicates that metabolic abnormalities associated with plasma NAD^+ and glutathione (GSH) depletion likely play fundamental roles underlying aging- and obesity-associated metabolic disorders [2,3]. Among the major organs in human body, the liver acts as a hub in whole-body NAD^+ homeostasis and is responsible for NAD^+ production. The production can occur through *de novo* synthesis from the essential amino acid tryptophan (the kynurenine pathway) or through conversion from different forms of vitamin B3 (NAD^+ precursors) in the salvage and Preiss-Handler pathways. However, a reduction in NAD^+ levels in age-related physiological states and pathological conditions involved in

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liver and central nervous system has emerged as a contributing factor to the pathogenesis of the diseases [4]. Thus, the therapeutic potential of restoration of NAD^+ levels has been investigated in a variety of pre-clinical models and followed by a series of human clinical trials, in which the efficacy of NAD^+-boosting therapeutics in human diseases are tested [5]. Moreover, GSH, which is synthesized from its constituent amino acids (glutamate, cysteine, and glycine), is the most abundant endogenous antioxidant preserving intracellular redox balance and is often depleted in pathogenesis with high oxidative load [6].

Our previous studies, which combined clinical data with multi-omics profiling, indicated that the plasma levels of NAD^+ and GSH are decreased in patients with non-alcoholic fatty liver disease (NAFLD) [3,7,8]. Based on our analysis, we proposed a therapeutic strategy by administration of Combined Metabolic Activators (CMA), which include NAD^+ precursors and GSH precursors, to simultaneously boost multiple deficient metabolic pathways to treat the disease [3,9]. We further tested CMA consisting of L-carnitine tartrate (LCAT), NAD^+ precursor nicotinamide riboside (NR), and GSH precursors L-serine and N-acetyl-L-cysteine (NAC), as individual metabolic activators in animal toxicology studies and placebo-controlled human clinical trials [10–14]. We have found the administration of CMA is safe and well-tolerance and improves the clinical outcomes of patients. For instance, CMA supplementation significantly decreased hepatic steatosis and improved liver functions in NAFLD patients [14], accelerated the recovery of Covid-19 patients [11], and improved cognitive functions of AD and PD patients [12,13].

One of the individual metabolic activators, NAC, is a well-known free-radical scavenger and a precursor of GSH. Although NAC could exert its direct antioxidant effect through physiological oxidants such as H_2O_2 and $O_2{\cdot}-$, the rate constants of the reaction are too low to make a significant contribution to oxidant scavenging [15,16]. On the other hand, it is often assumed that the antioxidative agency of NAC can be explained by its ability to act as a source of cysteine for increased GSH biosynthesis. Moreover, it has been reported that NAC was considerably less effective than cysteine at supporting GSH production [17]. Thus, whether NAC could be replaced with cysteine in CMA is a crucial question to be addressed in future clinical studies. Particularly, the difference in terms of the effects on global metabolism and safety profiling regarding the administration need to be further evaluated. Furthermore, recent studies on NAD^+ boosting have suggested that niacin and nicotinamide could be used as gold standards for other NAD^+-boosting strategy [18]. Therefore, a better understanding of metabolic differences after the administration of CMAs with different CMA constituents (namely formula with NAC or cysteine, and with or without NAD^+ precursor) can improve our knowledge in restoring NAD^+ and GSH levels when treating human diseases associated with metabolic abnormalities and can guide the development of different CMAs in clinical practices.

In this context, we studied the acute effect of placebo-controlled CMA administration with different constituents in well-characterized healthy participants and performed longitudinal untargeted-metabolomics profiling of plasma before and after administration. We also investigated the safety profile of CMA with cysteine. We further characterize the dynamic metabolic response to different CMA formulas, including L-serine, LCAT, NAC or cysteine, with or without NAD^+ precursors, including nicotinamide and flush-free niacin (FFN), by eliminating the effect of the fasting.

2. Results

We performed a one-day human study to investigate the acute effect and differential response of CMA with different formulas. In the study, 70 healthy subjects without any medication were recruited (age range of 18–47 years old, a body mass index (BMI) of 23.87 ± 3.43 kg/m^2, and 50% are male) and randomly assigned to receive CMA or placebo (Fig. 1A, Dataset S1, Table S1&S2). These CMA formulas were made up of L-serine, LCAT, cysteine or NAC, and with or without NAD^+ precursor (nicotinamide or flush-free niacin (FFN)). All of the subjects had the same breakfast at 08:00. After breakfast, the first blood samples were collected at 09:00 (T1) and followed by CMA supplementations or placebo. During the day, five additional blood samples were collected at 10:00 (T2), 11:00 (T3), 12:00 (T4), 14:00 (T5), and 16:30 (T6), respectively. During the study, none of the participants eat or drink anything except water. To assess the metabolic status of participants and evaluate the impact of CMA supplementation on clinical measures, we analyzed the plasma levels of aspartate transaminase (AST), alanine aminotransferase (ALT), C-reactive protein (CRP), triglycerides (TG), glucose, total cholesterol (TC), creatinine, and uric acid before and after the study.

Subjects supplemented by different CMA formulas did not show significant differences (one-way ANOVA, $p < 0.05$) in plasma levels of AST, ALT, CRP, TC, creatinine, and uric acid between baseline and endpoint of the study (Fig. 1B, Table S1&S2). We observed that plasma TG level was significantly decreased in groups treated with Formula 1 (CMA_F1, a mixture consisting of L-serine, LCAT, and NAC) (Fold Change (FC) = 0.52, $p = 0.047$), Formula 3 (CMA_F3, a mixture consisting of L-serine, LCAT, NAC, and nicotinamide) (FC = 0.58, $p = 0.032$), and Formula 4 (CMA_F4, a mixture consisting of L-serine, LCAT, cysteine, and nicotinamide) (FC = 0.63, $p = 0.017$) between baseline and endpoint of study (Fig. 1C, Table S1&S2). Plasma glucose level was significantly decreased in the group treated with Formula 6 (CMA_F6, a mixture consisting of L-serine, LCAT, cysteine, and FFN) (FC = 0.89, $p = 0.004$) during the period of the study. In addition, no side effects were reported in the CMA-treated groups (Dataset S1, Table S1&S2). Thus, we observed that administration of all CMA formulas was well-tolerated and no toxic effect was seen in the subjects participated in the study.

2.1. Altered metabolic pathways associated with CMA supplementation with cysteine or NAC

To characterize the dynamics of metabolic response to CMA administration and determine the differential response to CMA formulas, we performed longitudinal untargeted-metabolomics on 417 plasma samples across 6 time points from 70 participants and measured the levels of 1142 metabolites (Dataset S2). After excluding metabolites with missing values in <50% of samples, a total of 1007 metabolites were analyzed (Dataset S3). Metabolites significantly affected by CMA administration were identified using ANOVA after normalization to their baseline levels. Compared to placebo group, we observed that CMA supplementation with different formulas induced different global metabolic changes with the widest changes by CMA_F1 followed by CMA_F2 (a mixture consisting of L-serine, LCAT, cysteine) (Fig. 1D).

We first investigated the dynamic alterations of metabolites that are associated with the administration of CMA_F1 and CMA_F2. Plasma levels of 81 and 136 metabolites were significantly higher (one-way ANOVA, FDR <0.05) and lower, respectively, at least at one of time-points in the CMA_F1-treated group. Similarly, plasma levels of 99 and 22 metabolites were significantly higher and lower at least once in CMA_F2-treated group, respectively (Dataset S3). Next, we examined the over-representation of these significantly altered metabolites in different metabolic pathways. As expected, in both CMA_F1 and CMA_F2 treated groups, we observed that all significantly altered metabolites in metabolic pathways associated with L-serine and LCAT (two common metabolic activators in the CMA_F1 and CMA_F2) were significantly boosted, including amino acids metabolism (glycine, serine, and threonine), fatty acid metabolism (amino fatty acid, branched-chain amino acids metabolism, short-chain acylcarnitine, and hydroxy acylcarnitine), and carnitine metabolism (Fig. 2A). Moreover, we observed that in both CMA treated groups, the plasma levels of metabolites involved in urea cycle, creatinine, and secondary bile acid metabolism were significantly increased, whereas metabolites involved in the pentose metabolism, fructose, mannose, galactose metabolism were significantly

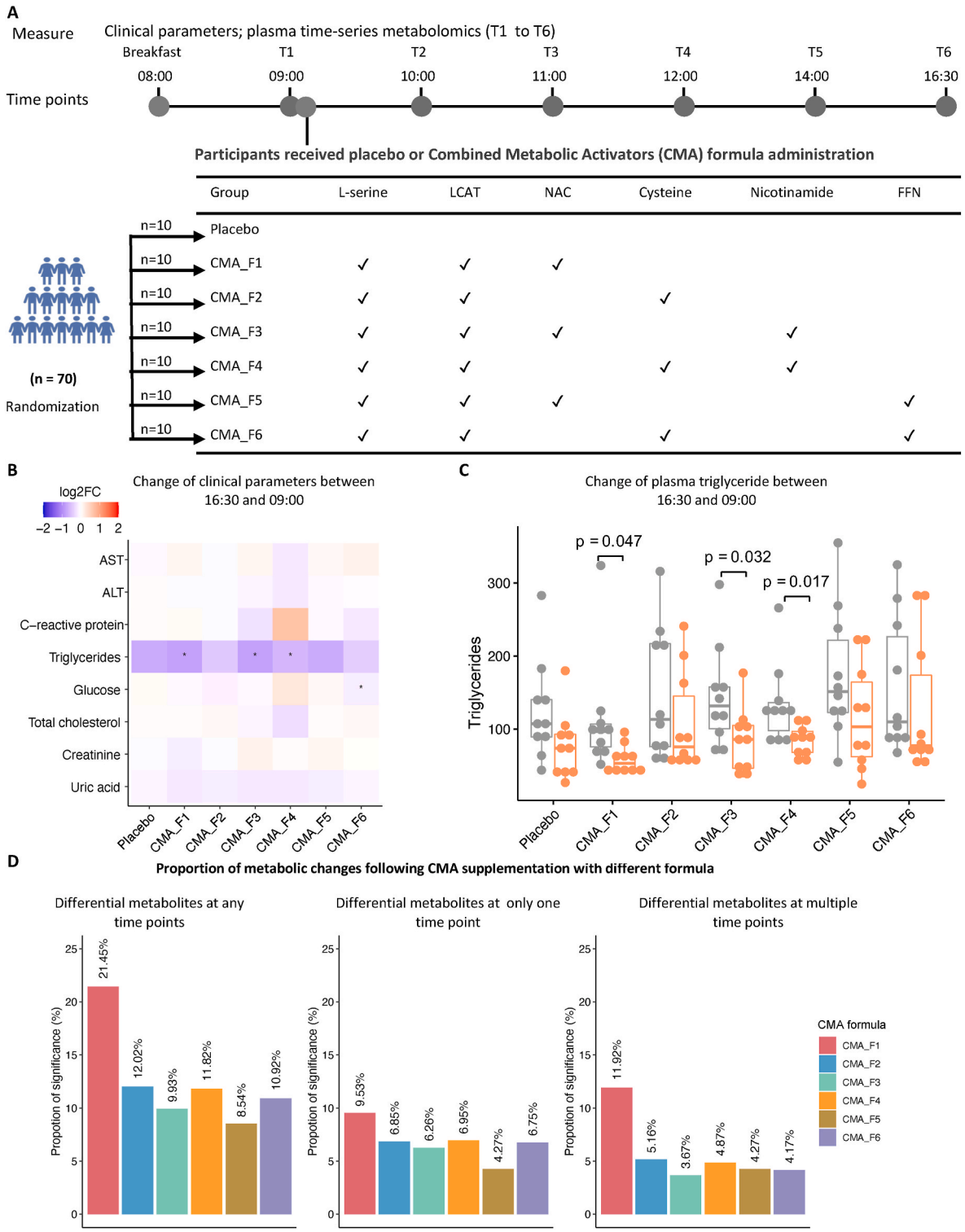

Fig. 1. Overview of study design and metabolic changes in response to CMA administration with different constituents. (**A**) 70 healthy participants were randomly assigned to receive placebo or one of six different Combined Metabolic Activator (CMA) formulas. The formulas tested were CMA_F1, CMA_F2, CMA_F3, CMA_F4, CMA_F5, and CMA_F6, each containing different combinations of L-serine, L-carnitine tartrate (LCAT), N-acetyl-L-cysteine (NAC), cysteine, nicotinamide, and flush-free niacin (FFN). Blood samples were collected at baseline (T1) and five subsequent time points after CMA administration for untargeted-metabolomics profiling. (**B**) Heatmap plot shows log2 fold changes (log2FC) of clinical parameters between baseline and endpoint of the study in each group including the CMA-treated groups and placebo group. Asterisk (*) denotes statistical significance ($p < 0.05$). The difference and p-value are derived from one-way ANOVA. (**C**) Boxplot shows the changes of plasma triglyceride levels between baseline and endpoint of the study in each group. (**D**) Bar-plot shows the global metabolic changes in response to different CMA supplementation as compared to placebo group. F, formula; T, time point; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

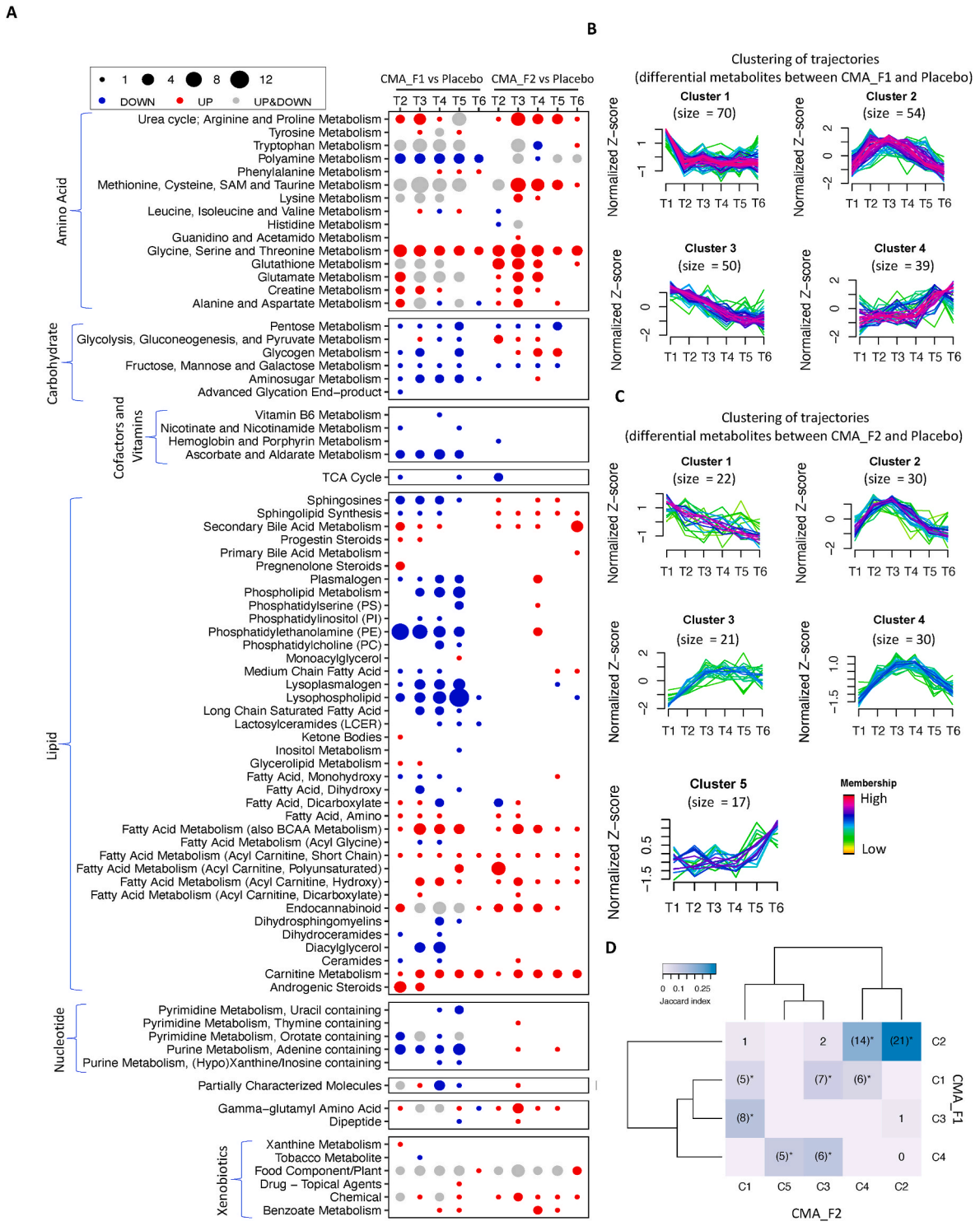

Fig. 2. Metabolic pathway perturbated by CMA supplementation with cysteine or NAC and longitudinal trajectories of altered metabolites (**A**) A dot-plot heatmap shows the altered pathway by CMA_F1 and CMA_F2 supplementation at each time point. The dot size represents the number of significantly dysregulated metabolites (one-way ANOVA, FDR <0.05) in the pathway between CMA-treated (CMA_F1 or CMA_F2) and placebo groups. The red, blue, and grey color of dot indicate there are only up-regulated, only down-regulated, and both up-regulated and downregulated metabolites in the pathway, respectively. (**B**) Time-course patterns clustered by significantly changed metabolites in response to CMA_F1 and (**C**) CMA_F2 supplementation using mFuzz (FDR <0.05), to illustrate the relative changes of metabolomics data. Trend lines are colour-encoded with red shades denoting high membership values of metabolites belonging to the time-series cluster. The *y* axis denotes the z-score of intensity for each metabolite. (**D**) Hierarchical clustering of Jaccard Index between cluster pairs from CMA_F1 and CMA_F2. Color scales represent the range of the Jaccard index. Asterisk indicates the statistical significance (hypergeometric test, $p < 0.05$) of the overlap between metabolites in any two clusters from the CMA-treated groups.

decreased. Interestingly, we observed that significantly altered metabolites involved in glycogen metabolism, sphingosines, and sphingolipid synthesis showed opposite changes in CMA_F1 and CMA_F2 treated groups (Fig. 2A). Metabolic pathways involved in the plasmalogen, phospholipid metabolism, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, and lactosylceramides were only affected in CMA_F1 treated group.

2.2. Longitudinal trajectories of altered metabolites with CMA supplementation with cysteine or NAC

In addition to investigation of the altered metabolic pathways, we used longitudinal pattern recognitions by fuzzy c-means clustering [19] to uncover specific patterns in significant altered metabolites in response to CMA_F1 and CMA_F2 administration. According to the number of tests supporting the optional clusters from 30 different NbClust testing methods [20], we identified four and five optimal clusters of longitudinal trajectories for CMA_F1 and CMA_F2 administration, respectively (Fig. 2B&C, Dataset S4). The analyses showed that, following CMA_F1 and CMA_F2 administration, 21 metabolites were shared between cluster 2 (size = 54) of CMA_F1-treated group and cluster 2 (size = 30) of CMA_F2-treated group, and the intersection is statistically significant (Fig. 2D; $p = 3.97 \times 10^{-22}$, hypergeometric test). Metabolites in these two clusters showed trajectories that increased in the beginning and returned to baseline at the end of the study. Notably, we observed that two of the substances in CMA_F1 and CMA_F2 (L-serine and cysteine) are members of these two clusters, respectively, suggesting dynamics of metabolites in these two clusters are closely associated with these two substances (Fig. 3A, B&C). Intriguingly, we observed that additional 14 metabolites in cluster 2 of CMA_F1-treated group were also significantly overlapped ($p = 3.63 \times 10^{-11}$, hypergeometric test) with cluster 4 (size = 30) of CMA_F2-treated group, in which these metabolites presented a relatively delayed increase before returning to baseline levels, including guanidinoacetate, propionylcarnitine (C3), cystathionine, and S-methylcysteine (Fig. 2C and 3B). In addition, carnitine (one common substance of CMA_F1 and CMA_F2 formulas) and its related metabolites (including deoxycarnitine, (S)-3-hydroxybutyrylcarnitine and acylcarnitine (C2)) reached to the peak levels after 3 h (T4) and slowly decreased afterward (Fig. 3A, B&C). These altered metabolites are grouped in cluster 4 (size = 39) of CMA_F1-treated group and cluster 3 (size = 21) of CMA_F2-treated group (Fig. 3B). Finally, some metabolites, such as mannitol/sorbitol, kynurenate, N−acetyl−isoputreanine, arabitol/xylitol, and methionine sulfone showed rapidly decrease following the supplementations, which are in cluster 3 (size = 50) of CMA_F1-treated group and cluster 1 (size = 22) of CMA_F2-treated group.

Taken together, we observed that L-serine, cysteine, and metabolites associated to these metabolic pathways presented similar dynamics after the administration of both CMA_F1 and CMA_F2, as we expected, showing a quick increase before returning to baseline at endpoint of the study. Several metabolic pathways involved in the lipid metabolism, however, were only affected by the administration of CMA_F1 (Fig. 2A). Notably, we observed that the plasma level of nicotinamide, a precursor of NAD^+ biosynthesis, rather than cysteine showed significant difference between CMA_F1 and CMA_F2 groups (Fig. 3C), suggesting that the conversion from NAC to cysteine might indirectly affect NAD^+ pool by decreasing the level of nicotinamide. In addition, some metabolites involved in cysteine metabolism (including hypotaurine, taurine, and cysteine-glutathione disulfide) and glycogen metabolism (maltotetriose and maltotriose) showed opposite trajectory in CMA_F1- and CMA_F2-treated groups (Fig. 3B and D).

2.3. Considering NAD^+ precursor as one of components of CMA

We further investigated the effect of CMA_F3 and CMA_F4 on plasma metabolomics by introducing nicotinamide as a metabolic activator (is also called niacinamide) into both CMA_F1 and CMA_F2 (Fig. 1A). The plasma level of nicotinamide, which kept consistent (stable) in the placebo group, presented dynamics with a quick increase within 1 h (T2) following the administration and then stayed relatively stable for 2 h (T2 to T4) before returning to baseline in both CMA_F3 and CMA_F4 groups (Fig. 4B). Notably, the plasma level of cysteine was found to be significantly higher in CMA_F4-treated group within the first 2 h compared to placebo group, while no significant difference was observed in the CMA_F3-treated group during the same time period. Interestingly, the cysteine level in the CMA_F3-treated group subsequently decreased over time and reached a level lower than baseline at T5. The plasma levels of L-serine and carnitine showed a similar trajectory as in the groups treated without nicotinamide (Fig. 3C and 4B). In addition, we found there were 100 and 120 metabolites significantly altered at least at one of the timepoints in the CMA_F3 and CMA_F4-treated groups compared to placebo group, respectively (Fig. 4C). Pathway analysis showed that metabolites involved in the pathways such as amino acid metabolism (glycine and serine), fatty acid metabolism (BCAA metabolism, short-chain acylcarnitine, and hydroxy acylcarnitine), carnitine metabolism, and nicotinate and nicotinamide metabolism, have significantly higher plasma levels in both CMA_F3 and CMA_F4-treated groups (Fig. 4D). However, metabolites involved in polyamine metabolism, pentose metabolism, vitamin B6 metabolism, dicarboxylate acids catabolism (fatty acid, dicarboxylate), and fructose, mannose, and galactose metabolism, have significantly lower plasma levels in both CMA_F3 and CMA_F4 groups (Fig. 4D).

Using longitudinal pattern recognitions, we identified four and five main clusters of distinct patterns for the significantly differential metabolites in response to CMA_F3 and CMA_F4 administration, respectively (Fig. 4E, Dataset S4). We observed that metabolites of cluster 3 (size = 28) in the CMA_F3-treated group and metabolites of cluster 2 (size = 26) in the CMA_F4-treated group are significantly overlapped ($p = 2.75 \times 10^{-23}$, hypergeometric test), presenting an immediate response to CMA_F3 or CMA_F4 administration (Fig. 4E). Of note, we found that both clusters included three of the metabolic activators, including L-serine, carnitine, and nicotinamide (Fig. 4F). With the administration of CMA_F4, the endpoint plasma levels of cysteine and several other metabolites such as N-acetyl-2-aminooctanoate*, threonine, and 2-aminobutyrate in cluster 4 (size = 25) was decreased after an increase at the beginning of the administration (Fig. 4B and 5A). In addition, we observed that metabolites of cluster 5 (size = 18) in the CMA_F4-treated group presented a slow decrease and returned to baseline trend, including some medium/long-chain dicarboxylic acid (for example, dodecenedioate (C12:1-DC)*, hexadecanedioate (C16-DC), and tetradecanedioate (C14-DC) which are generated during ω-oxidation) and metabolites involved in kynurenine pathway such as kynurenate and 8-methoxykynurenate (Fig. 5A&B). Similarly, long-chain dicarboxylic acid octadecanedioate (C18-DC) in cluster 1 (size = 17) was decreased slowly following CMA_F3 supplementation and returned to baseline.

The pathway (so called Preiss-Handler Pathway) involving nicotinic acid (is also called niacin) is critical for NAD^+ production and the pharmacological role of niacin has been extensively studied in rodents and human clinical trials [21,22] (Fig. 4A). Hence, we introduced niacin as metabolic activator into CMA_F1 (new formula called CMA_F5) and into CMA_F2 (new formula called CMA_F6) to investigate the metabolic alterations that occur in response to the supplementations. Considering the previously reported adverse flush response of niacin, we used flush-free niacin (FFN) instead of niacin in the current study. As expected, FFN introduction did not significantly boost the plasma level of nicotinamide until T6 in the CMA_F6-treated group due to its slow release (Fig. 5C). Similarly, pathway analyses revealed that some metabolites involved in the lipid metabolism pathways such as plasmalogen, phospholipid, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), and glycogen metabolism were only significantly increased at T6 in the CMA_F6-treated group (Fig. 5D). Moreover,

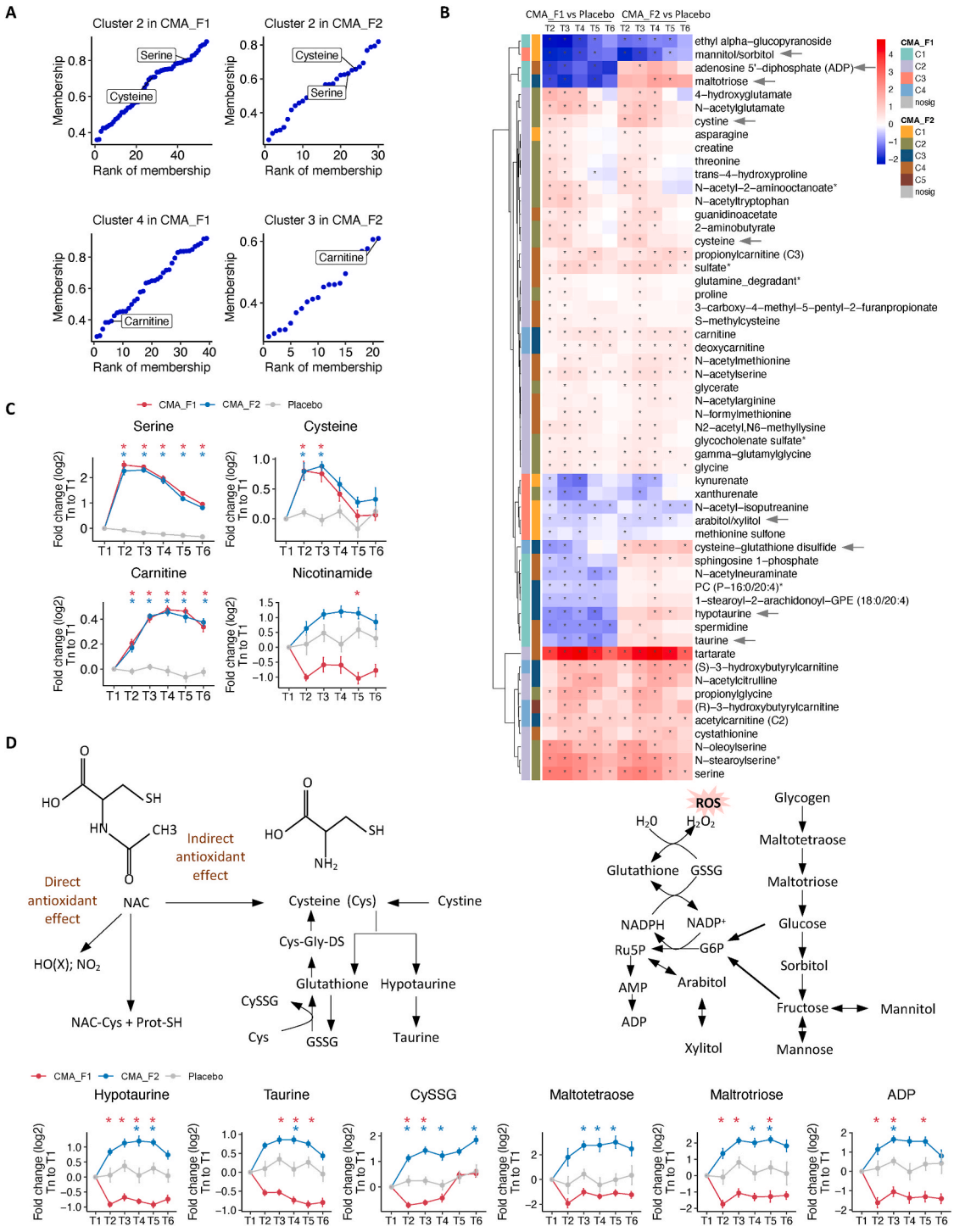

Fig. 3. Kinetic of each metabolic activator and of that metabolite involved in cysteine and glycogen metabolism (**A**) Dot-plots show the membership of plasma CMA substances in corresponding longitudinal trajectory clusters, dot indicates the metabolites in the cluster, the x-axis denotes the rank of membership of all metabolites in the cluster and y-axis denotes the value of membership. (**B**) Heatmap represents the log2 fold change relative to placebo at each timepoint for significantly altered metabolites in both CMA_F1 and CMA_F2 groups. Metabolites Only altered at least two time points between T2 and T4 were presented. The longitudinal trajectory cluster of each metabolite is indicated on the left side of the heatmap. The color indicates log-transformed fold change between CMA-treated group and placebo at certain timepoint after normalization to their corresponded baseline level, red and blue represent up- and down-regulation of metabolites in supplementation group compared to placebo group. The single asterisk behind a metabolite indicates that the metabolite was annotated based on in silico prediction [38]. (**C**) Longitudinal trajectories of plasma CMA substances (including serine, cysteine, and carnitine) and nicotinamide in response to the CMA supplementation. The dots represent the mean log2 fold change relative to baseline, with bars for the standard error of the mean (SEM). Asterisk (*) denotes statistical significance (one-way ANOVA, FDR <0.05). (**D**) Schematic of differential altered metabolites in cysteine metabolism (left) and glycogen metabolism (right) by CMA_F1 and CMA_F2. NAC, N-acetyl-L-cysteine. Cys, cysteine. Cys-Gly-DS, cysteinyglycine disulfide*. CySSG, cysteine-glutathione disulfide. GSSG, Glutathione disulfide. G6P, glucose 6-phosphate. Ru5P, ribulose 5-phosphate.

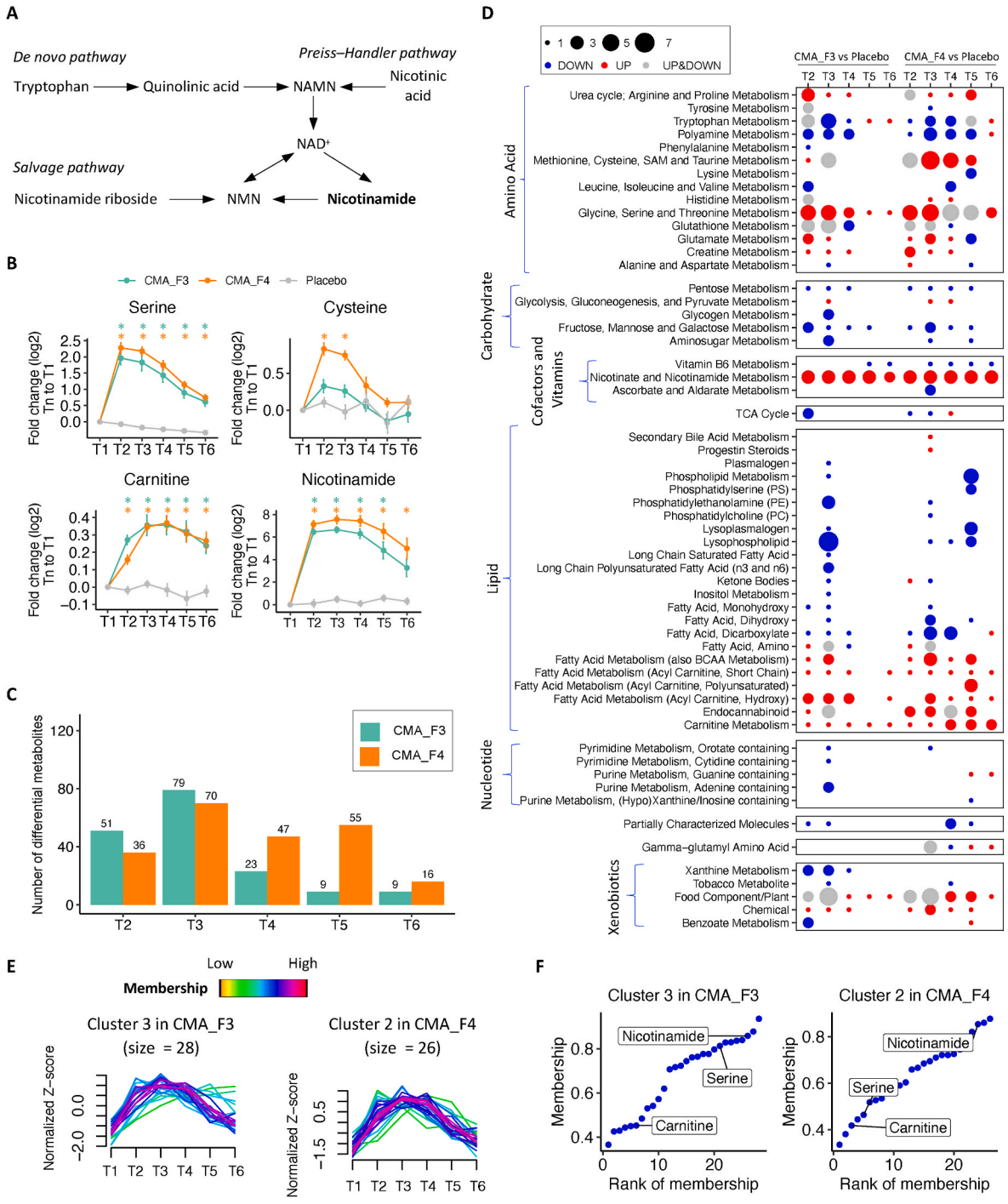

Fig. 4. Considering NAD $^{+}$ precursor as one of components of CMA (**A**) NAD $^{+}$ biosynthesis pathways either *de novo* from tryptophan or via salvaging from different forms of vitamin B_3 (so called NAD^{+} precursors) including nicotinic acid (niacin), nicotinamide riboside, and nicotinamide. NAMN, niacin mononucleotide. NMN, nicotinamide mononucleotide. (**B**) Relative changes of serine, cysteine, carnitine and nicotinamide plasma levels compared the baseline in the supplementation (CMA_F3 and CMA_F4) and placebo group at each time point. The dots represent the mean log2 fold change relative to baseline, and the bars are the standard error of the mean (SEM). (**C**) Bar-plot shows the number of differential metabolites (one-way ANOVA, FDR <0.05) between CMA supplementation groups (CMA_F3 and CMA_F4) and placebo group at each timepoint. (**D**) Dot-plot shows the altered pathway by CMA_F3 and CMA_F4 supplementation at each time point. The dot size represents the number of significantly dysregulated metabolites (one-way ANOVA, FDR <0.05) in the pathway between CMA-treated (CMA_F3 or CMA_F4) and placebo groups. The red, blue, and grey color of dot indicate there are only up-regulated, only down-regulated, and both up-regulated and downregulated metabolites in the pathway, respectively. (**E**) Two example clusters of longitudinal trajectories for metabolites exhibiting a very rapid response reaching a maximum level following supplementation (CMA_F3 and CMA_F4) and returning to baseline at the endpoint of the study. (**F**) Dot-plot shows the membership of plasma CMA substances, dot indicates the metabolites in the cluster, the x-axis denotes the rank of membership of all metabolites in the cluster, and the y-axis denotes the value of membership.

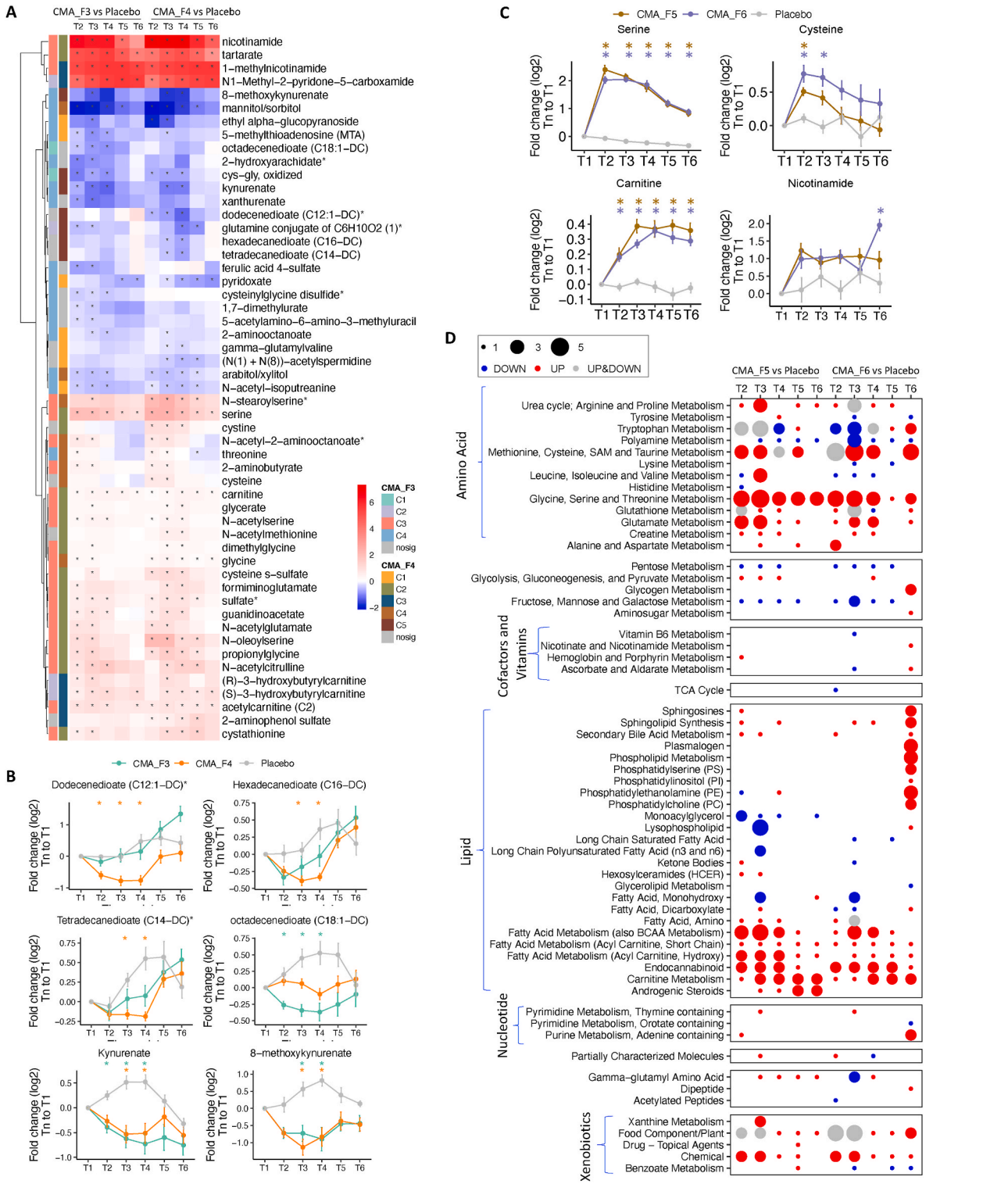

Fig. 5. Altered metabolic pathways associated with CMA supplementation with nicotinamide or FFN (**A**) Heatmap shows the log2 fold change relative to placebo at each timepoint for significantly altered metabolites in both CMA_F3 and CMA_F4 groups, only metabolites altered at least two time points between T2 and T4 were presented. The single asterisk behind a metabolite indicates that the metabolite was annotated based on in silico prediction [38]. The longitudinal trajectory cluster of each metabolite is indicated on the left side of the heatmap. The color indicates log2 fold change, red and blue represent up- and down-regulation of metabolites in supplementation group compared to placebo group. Asterisk (*) denotes statistical significance (one-way ANOVA, FDR <0.05). (**B**) Longitudinal trajectories of plasma metabolites involved in dicarboxylic acid oxidation in response to CMA supplementation with additional nicotinamide supplementation (CMA_F3 and CMA_F4 groups). (**C**) Longitudinal trajectories of plasma CMA substances in response to CMA_F5 and CMA_F6 supplementations. The dots represent the mean log2 fold change relative to baseline and the bars are the standard error of the mean (SEM). (**D**) A dot-plot shows the altered pathway by CMA_F4 and CMA_F5 supplementation at each time point. The dot size represents the number of significantly dysregulated metabolites (one-way ANOVA, FDR <0.05) in the pathway between CMA-treated (CMA_F4 or CMA_F5) and placebo groups. The red, blue, and grey color of dot indicate there are only up-regulated, only down-regulated, and both up-regulated and downregulated metabolites in the pathway, respectively.

metabolic pathways involved in amino acids metabolism (including glycine, serine, threonine, glutamate, and creatine), fatty acid metabolism (BCAA metabolism, short-chain acylcarnitine, and hydroxy-acylcarnitine), endocannabinoid, carnitine metabolism were boosted by both CMA_F5 and CMA_F6 administrations (Fig. 5D). The metabolites involved in pathways such as polyamine metabolism, pentose metabolism, fructose, mannose, and galactose metabolism were significantly decreased by the administration of those two CMAs.

Longitudinal trajectories analyses revealed that some metabolites including cysteine, cysteine s-sulfate, and thioproline (in cluster 2 of CMA_F5 group and cluster 3 of CMA_F6 group) displayed quick decrease after reaching the peak at T2, which showed a relatively slow decrease in the CMA_F6-treated group (Fig. 5C and 6A&B, Dataset S4). In addition, some metabolites such as hypotaurine, taurine, ADP, maltotriose, maltose, and sphingosine kept increasing from baseline in both CMA_F5 and CMA_F6 treated groups. However, the increase only became statistically significant at T6 in the CMA_F6 group (Dataset S3).

3. Discussion

Based on our previous studies combing in-depth multi-omics profiling and systems biology analyses, we found that supplementation of CMA consisting of GSH and NAD^+ precursors could activate the altered metabolic pathways in pathogenesis of multiple human diseases, including NAFLD, COVID-19, AD, and PD [3,10–14]. In this study, we performed a one-day placebo-controlled human kinetic study to explore potential substitution of NAC with cysteine itself in the CMA through longitudinal untargeted-metabolomics profiling in healthy participants. On the one hand, NAC is the limiting precursor in the formulation of the antioxidant GSH and is the most widely used oxidant scavenger in both *in vitro* and *in vivo* experiments, as well as in clinical studies [9,23]. On the other hand, cysteine is the second of most widely used endogenous metabolic modulator and it has shown promising effects in clinical trials for several diseases [9]. Given the fact that NAC exerts its indirect antioxidant effect via conversion to cysteine, it is worth investigating the possibility of introducing cysteine instead of NAC in CMA for supporting GSH synthesis. There are also limitations in the use of NAC as a dietary supplementation since it has been approved as a drug in many different countries, even though it is widely sold over the counter [24]. The U.S. Food and Drug Administration (FDA) recently has warned all companies selling NAC as a dietary supplement [25].

In our study, we showed that the administration of all CMA formulas significantly boosted the metabolic pathways associated with L-serine and LCAT, which are common components of the CMA formulas. Multiple studies have demonstrated that limited serine availability is one of the landmark metabolic features of human such as NAFLD, COVID, AD,

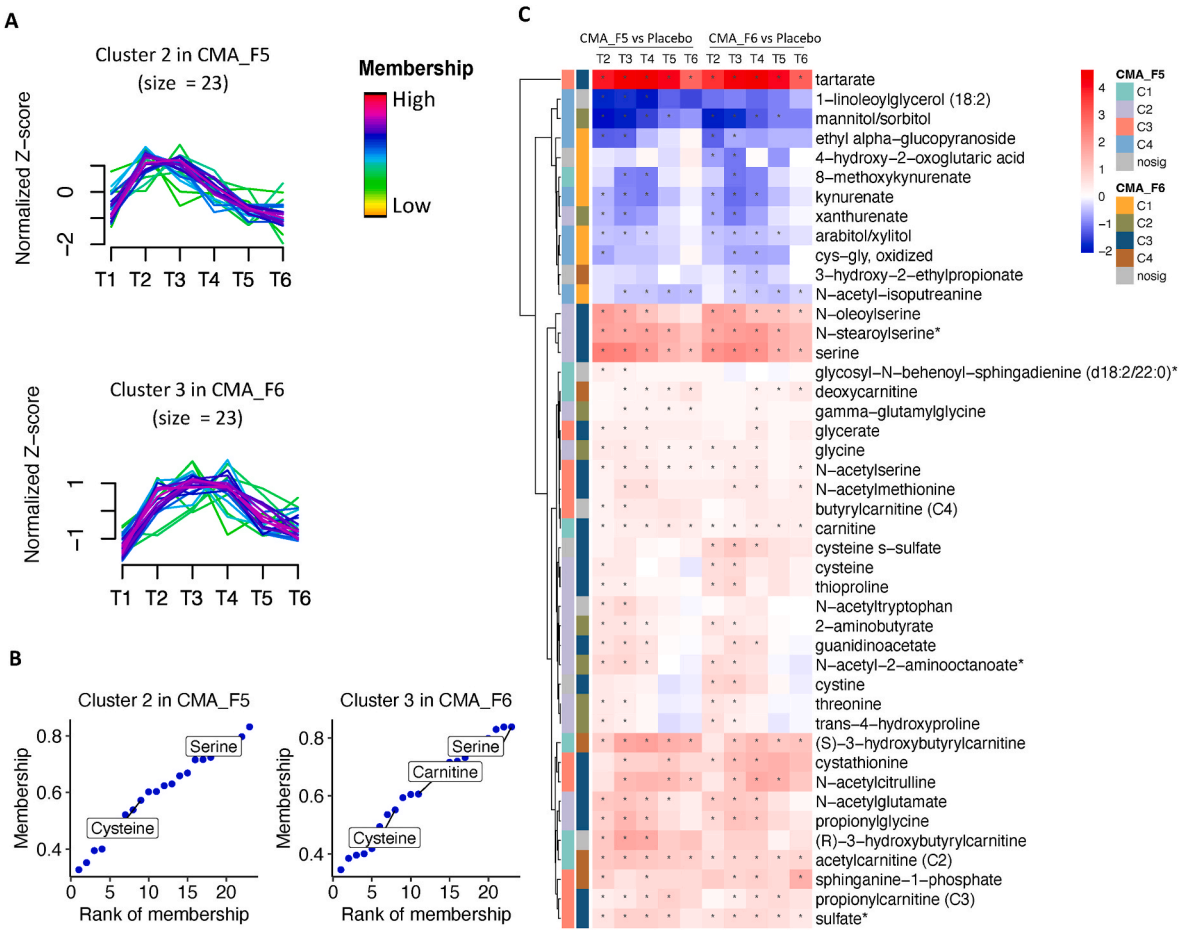

Fig. 6. Longitudinal pattern of metabolites associated with metabolic activators followed by administration of CMA with FFN (**A**) Two example clusters of longitudinal trajectories for metabolites exhibiting a very rapid response reaching a maximum level following supplementation (CMA_F5 (upper) and CMA_F6 (Lower)) and returning to baseline at the endpoint of the study. (**B**) Dot-plot shows the membership of CMA substances, dot indicates the metabolites in the cluster, the x-axis denotes the rank of membership of all metabolites in the cluster, and the y-axis denotes the value of membership. (**C**) Heatmap shows the log2 fold change relative to placebo at each timepoint for significantly altered metabolites in both CMA_F5 and CMA_F6 groups, only metabolites altered at least two time points between T2 and T4 were presented. The clusters of trajectories are indicated on the left side of the heatmap. The color indicates log-transformed fold change, red and blue represent up- and down-regulation of metabolites in supplementation group compared to placebo group, respectively. The single asterisk behind a metabolite indicates that the metabolite was annotated based on in silico prediction [38]. Asterisk (*) denotes statistical significance (one-way ANOVA, FDR <0.05).

and PD [3,26]. Serine metabolism has an impact on the mitochondrial function and dynamics [27]. Dietary L-serine supplementation may serve as an essential therapeutic strategy for restoring mitochondrial dysfunction induced by serine deficiency [26,28]. Carnitine is a carrier molecular that delivers substrates such as long or very-long fatty acids across the mitochondrial membrane for oxidation and thus required in maintaining energy metabolism. Plasma levels of kynurenate and 8-methoxykynurenate were increased in COVID-19 patients [29] and are significantly positively associated with increased liver fat in patients with NAFLD [3]. In this study, we showed that the plasma levels of kynurenate and 8-methoxykynurenate were significantly decreased in all CMA treated groups.

In addition, we found that the plasma level of cysteine between CMA_F1 (consisting of L-serine, LCAT, and NAC) and CMA_F2 (consisting of L-serine, LCAT, cysteine) did not show significant difference rather than similar trajectories at first 3 h, suggesting that both NAC and cysteine administration can similarly elevate the cysteine level for GSH biosynthesis. Interestingly, we observed opposite trends in the levels of cysteine degradation metabolites, such as hypotaurine and taurine, during the supplementation of CMA_F1 and CMA_F2. Taurine and its precursor hypotaurine have been reported to be involved in the NAD^+ salvage [30] and play an important role in reducing oxidative stress and inflammation induced by exercise and inflammatory diseases [31,32]. Moreover, several studies have shown that taurine is efficient in managing cholesterol metabolism and reducing plasma and liver cholesterol concentrations [33]. Notably, we found that the plasma level of nicotinamide, a NAD^+ precursor in the salvage pathway, presented different kinetics between the CMA_F1 and CMA_F2 groups without the supplementations of any NAD^+ precursors. Our analysis suggested that administration of the CMA_F1 or CMA_F2 activate multiple pathways and additional NAD^+ precursor may be needed in CMA for optional clinical effect.

A growing number of studies demonstrated that NAD^+ depletion might be a contributing factor to aging and related pathological conditions, such as NAFLD, COVID, AD, and PD [2,3,11,14,18]. NAD^+ boosting strategies, either stimulation of its synthesis or inhibition of its consumption, have been widely explored in a number of preclinical models and clinical studies of human diseases [34]. Based on the promising effect of NR (a NAD^+ precursor) administration in the previously tested CMA in the preclinical and clinical studies, here we investigated the global effect of other NAD^+ precursors including nicotinamide or FFN in the CMA with NAC or cysteine to further evaluate the metabolic differences compared to the placebo group. In this study, we found that incorporation of nicotinamide rather than FFN into both CMA_F1 and CMA_F2 significantly boosted the plasma level of nicotinamide and its related products including 1-methylnicotinamide and N1-Methyl-2-pyridone-5-carboxamide. Our analysis suggested that nicotinamide as a metabolic activator may provide sufficient substrate for NAD^+ to activate mitochondria as the administration of NR in the CMA [14,35–37]. Nevertheless, further investigation will be required to characterize more detailed molecular mechanism underlying the administration of CMA with nicotinamide. Interestingly, several metabolites involved in fatty acid ω-oxidation showed significant decrease in the groups (CMA_F3 and CMA_F4) with nicotinamide on the basis of L-serine, carnitine, and cysteine or NAC. However, we did not observe similar trend of these metabolites in the CMA formulas with FFN (CMA_F5 and CMA_F6).

In summary, these results suggested that administration of CMA with cysteine is safe and well-tolerated in participants in the study. In the presence of NAD^+ precursors, the untargeted metabolomics profiling further revealed a similar response to CMA formulas with NAC or cysteine, suggesting that NAC could be replaced with cysteine in the CMA. Future efforts will provide additional understanding of how patients with metabolic disorders can be benefited from the CMA supplementation with different metabolic activators.

4. Material and methods

4.1. Study design

We performed the one-day kinetic study by recruiting 70 healthy subjects without any medicine at the Umraniye Training and Research Hospital, University of Healthy Sciences, Istanbul, Turkey. Informed consent was obtained from all participants involved in this study. The safety of the participants and the risk-benefit analysis were overseen by an independent external data-monitoring committee. The trial was conducted in accordance with Good Clinical Practice guidelines and the principles of the Declaration of Helsinki. The study was approved by the ethics committee of Istanbul Medipol University, Istanbul, Turkey (under no. E-10840098-772.02-2670).

The inclusion criteria are that the participants of both genders (female and male) should be over 18 years of age, healthy without any medication and obesity. The participants who have known diseases, allergy for substances used in the study, and over alcohol consumption are excluded. The main characteristics of the subjects are summarized in Tables S1 and S2.

4.2. Randomization, interventions and follow-up

Participants were randomly assigned to receive 6 different CMA formulations or placebo. The composition of different CMA formulas was shown in Fig. 1A. Patient information (including patient number, date of birth, and initials) was entered into the web-based randomization system, and the randomization codes were entered into the electronic case report form. All clinical staff were blinded to treatment, as were the participants.

All participants had breakfast at 8:00 in the morning and were followed up for one day. Blood samples were collected at 9:00 (Time point 1, T1). Then, the participants orally took combined metabolic activators with certain formulas (CMAs) or placebo (Fig. 1A). Both CMAs and placebo were provided in powdered form in an identical plastic bottle containing a single dose to be dissolved in water. Afterward, blood samples were collected again at 10:00 (T2), 11:00 (T3), 12:00 (T4), 14:00 (T5) and 16:30 (T6). Each dosage of CMA dose contained 12.35 g serine, 2.55 g cysteine or NAC, 3.73 g LCAT and 1 g FFN, or nicotinamide. Clinical parameters (e.g., ALT, AST and uric acid) were measured in T1 and T6.

4.3. Untargeted metabolomics analysis

Plasma samples were collected at 9:00 (T1), 10:00 (T2), 11:00 (T3), 12:00 (T4), 14:00 (T5), 16:30 (T6) 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 to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) and centrifuged. The resulting extract was divided into four fractions: one each for analysis by ultraperformance liquid chromatography–tandem mass spectroscopy (UPLC-MS/MS) with positive ion-mode electrospray ionization, UPLC-MS/MS with negative ion-mode electrospray ionization, and gas chromatography–mass spectrometry; one fraction was reserved as a backup. An in-depth description of these processes used by Metabolon is described in Ref. [38].

4.4. Fuzzy c-mean clustering

In order to reveal time-course patterns in statistically significant metabolites responses to CMA supplementation, we used fuzzy c-means clustering approach to perform longitudinal pattern recognitions [19].

We first used NbClust method [20], available in NbClust R package (version 3.0), to determine the optimal number of clusters of metabolites that significant were altered in subjects with CMA supplementation as compared to those in placebo group with similar longitudinal trend. In this study, the number of appropriate clusters was chosen based on the support from the most NbClust testing methods (Euclidean distance, Ward method clustering, from 4 to 10 clusters. In total, more than 30 testing methods were used). Fuzzy c-mean clustering was performed using the 'Mfuzz' (version 2.48.0) R package after normalization of the data.

4.5. Statistical analysis

One-way ANOVA was used to identify the difference in clinical parameters between different groups or time points. The metabolite profiles with missing values in more than 50% of samples were removed. One-way ANOVA was used to detect the metabolites which showed significantly different changes between groups at each time point, in which all data points were normalized into fold changes by comparing to their corresponding baseline value which is time point T1. In all analyses, missing values were removed in a pairwise manner. The p-values were adjusted by Benjamini & Hochberg method. Metabolites with a false-discovery rate of 5% (FDR <0.05) were considered statistically significant. All statistical analyses were performed by R language (version 4.0.3).

Author contributions

Conceptualization, **A.M., C.Z.**; Methodology, Software, and Formal analysis, **H.Y. and X.L.**; Investigation, **H.Y., X.L., G.O., H.D., and C.Z.**; Writing - Original Draft, **H.Y.**; Writing - Review & Editing, **H.Y., X.L., H. J., H.T., J.N., G.O., H.D., M.U., J.B., C.Z., and A.M.**; Funding Acquisition, **A.M.**; Supervision, **C.Z. and A.M.**

Declaration of competing interest

A.M., J.B., and M.U. are the founder and shareholders of ScandiBio Therapeutics and they filed a patent application on the use of CMA to treat patients with different diseases. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.05.013.

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