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Methionine transsulfuration pathway is upregulated in long-lived humans

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ABSTRACT

Available evidences point to methionine metabolism as a key target to study the molecular adaptive mechanisms underlying differences in longevity. The plasma methionine metabolic profile was determined using a LC-MS/MS platform to systematically define specific phenotypic patterns associated with genotypes of human extreme longevity (centenarians). Our findings demonstrate the presence of a specific plasma profile associated with human longevity characterized by an enhanced transsulfuration pathway and tricarboxylic acid (TCA) cycle intermediates, as well as a reduced content of specific amino acids. Furthermore, our work reveals that centenarians maintain a strongly correlated methionine metabolism, suggesting an improved network integrity, homeostasis and more tightly regulated metabolism. We have discovered a particular methionine signature related to the condition of extreme longevity, allowing the identification of potential mechanisms and biomarkers of healthy aging.

1. Introduction

Methionine is an essential proteinogenic amino acid selected and incorporated to the universal genetic code based on their functional properties and in response to the appearance of biospheric molecular oxygen during early evolution [1,2]. In fact, molecular oxygen demanded early organisms to incorporate additional amino acids with increased redox properties into the genetic code like lysine, histidine, phenylalanine, cysteine, tyrosine, tryptophan, and selenocysteine as adaptation to preserve aerobic life [1]. Interestingly, the protein content of the sulphur amino acids methionine and cysteine keeps a relationship with animal longevity. Thus, the longer the animal longevity, the lower the methionine [3–6] and cysteine [7] protein content, probably as adaptive response [8] to the lower oxidative stress also present in long-lived animal species [8–10]. Later, these observations were extended to the free tissue methionine content in diverse long-lived animal species [11,12]. Reinforcing these findings, methionine restriction is a nutritional intervention that extend animal longevity [8,9,13]. Consequently, available evidences point to methionine metabolism as a key target to study the molecular adaptive mechanisms underlying differences in longevity.

Beyond its function in several intracellular processes, methionine is central in a complex metabolic pathway which can be divided in three parts: methionine cycle, the transsulfuration pathway, and polyamine biosynthesis [14,15] (Fig. 1). Methionine is an essential sulphur-containing metabolite that is mainly metabolized through the transmethylation pathway or the methionine cycle. Briefly, methionine is converted to the universal methyl donor S-adenosylmethionine (SAM), which upon donation of one methyl group is converted to S-adenosylhomocysteine (SAH). SAH is hydrolyzed into homocysteine,

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which can be used to regenerate methionine via betaine or folate cycle. However, homocysteine can enter into the transsulfuration pathway, and be sequentially converted into cystathionine and cysteine in a series of reactions catalyzed by enzymes that use vitamin B6 as cofactor. Finally, this cysteine can be directed to the glutathione synthesis or, via several metabolic reactions, to the synthesis of taurine. Significantly, manipulation of each of these branches affects longevity in diverse animal models [13].

As a consequence of these observations, it is plausible to postulate that long-lived humans (like centenarians) significantly delay or in several cases even avoid age-associated diseases because they express and have a specific methionine metabolic phenotype. Up to date no targeted metabolomics analysis investigating differences in the plasma methionine metabolome of exceptionally long-lived humans have been reported. Blood plasma is the major carrier of metabolites in the body [16]. The composition of this biological fluid is well-known, even it is continuous change, reflecting physiological states in health and disease [17,18]. Although metabolomics data interpretation is often challenging and mostly descriptive, it is more than a source of potential biomarkers and metabolomic signatures associated with specific states, such as longevity [11]: it also allows the identification of new mechanistic pathways or targets that might lead to healthier and longer lives. To this end, we have designed a study to detect and quantify a panel of metabolites including 36 different molecular species: a) methionine and its related metabolites, including the intermediates of the transmethylation pathway SAM, SAH and homocysteine; betaine and spermidine as metabolites involved in the regeneration of methionine plasma levels; the intermediates of the transsulfuration pathway cysteine and cystathionine; taurine and glutathione as downstream metabolites of the transsulfuration pathway; and vitamin B6 metabolites pyridoxal, pyridoxal-5′-phosphate and pyridoxamine, as cofactors of the transsulfuration enzymes; b) additional amino acids including 7 non-polar amino acids (alanine, glycine, leucine/isoleucine, phenylalanine, proline, tyrosine and valine), 4 polar uncharged amino acids (asparagine, serine, threonine and tryptophan), 1 polar negatively charged amino acid (glutamate) and 2 polar positively charged amino acids (arginine and histidine); c) TCA cycle-related metabolites, including pyruvate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate and malate; and d) methionine-derived lipid intermediates such as choline and carnitine. The selection of specific metabolites allows to step back from the view of methionine as a solely metabolite associated to longevity and obtain a global view of methionine metabolism modulation in centenarians. However, this also come up with other limitations due to the intrinsically included bias, as other metabolites that might be metabolically related to methionine might be left from the analyses, whereas others less relevant might be included. The plasma metabolites profile was determined using a LC-MS/MS platform to systematically define specific phenotypic patterns associated with genotypes of human extreme longevity.

2. Results

2.1. Centenarians have a unique methionine-related metabolites plasma profile

In order to determine whether plasma methionine and its related metabolites concentration differed among adult, aged and centenarian individuals, multivariate statistics were applied. Non-supervised principal component analysis (PCA) suggested a different specific plasma methionine metabolites profile in centenarians (Fig. 2A), capable to explain 48.7% of sample variability. A hierarchical clustering of the samples represented by a heat map revealed that centenarian's unique plasma profile of methionine-related metabolites was different from those of adults and aged individuals (Fig. 2B). These results were confirmed by performing a supervised analysis, such as partial least squares discriminant analysis (PLS-DA), which showed that plasma metabolite content is a good model to identify the different groups. The model was trained using 60% of the samples and tested using the

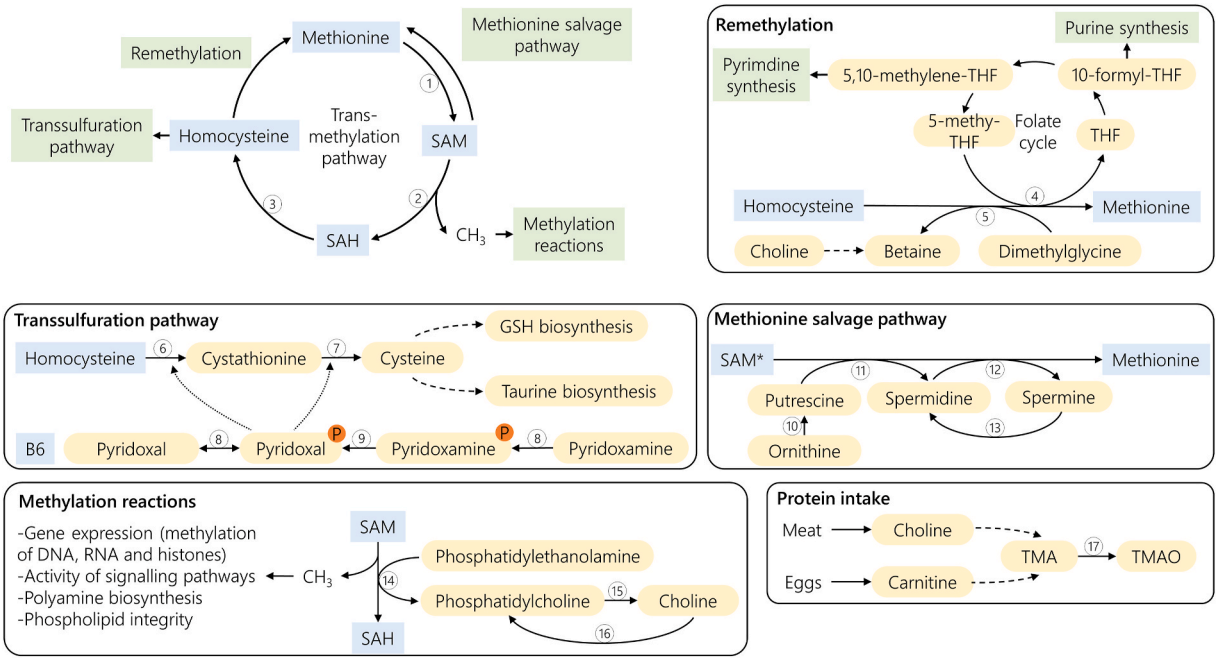

Fig. 1. Methionine metabolism and related metabolic processes. Numbers refer to enzymes: **1**, Methionine adenosyltansferase Iα/IIα (MAT1A/MAT2A); **2**, Methyltransferases (MTs); **3**, Adenosylhomocysteinase-like 1 (AHCYL); **4**, Methionine synthase (MS); **5**, Betaine-Homocysteine S-methyltransferase (BHMT); **6**, Cystathionine-β-synthase (CBS); **7**, Cystathionine-γ-lyase (CTH); **8**, Pyridoxal kinase (PDXK) or pyridoxal phosphatase (PDXP); **9**, Pyridoxine 5′-phosphate oxidase (PNPO); **10**, Ornithine decarboxylase (ODC1); **11**, Spermidine synthase (SRM); **12**, Spermine synthase (SMS); **13**, Spermine oxidase (SMOX); **14**, Phosphatidylethanolamine N-methyltransferase (PEMT); **15**, Phospholipase C; **16**; CPD-choline pathway; **17**, Flavin mono-oxygenase 3 (FMO) in the liver. **SAM*** represents S-adenosyl-3-(methylsulfanyl)propylamine, synthesised from SAM via S-adenosylmethionine decarboxylase proenzyme (AMD1).

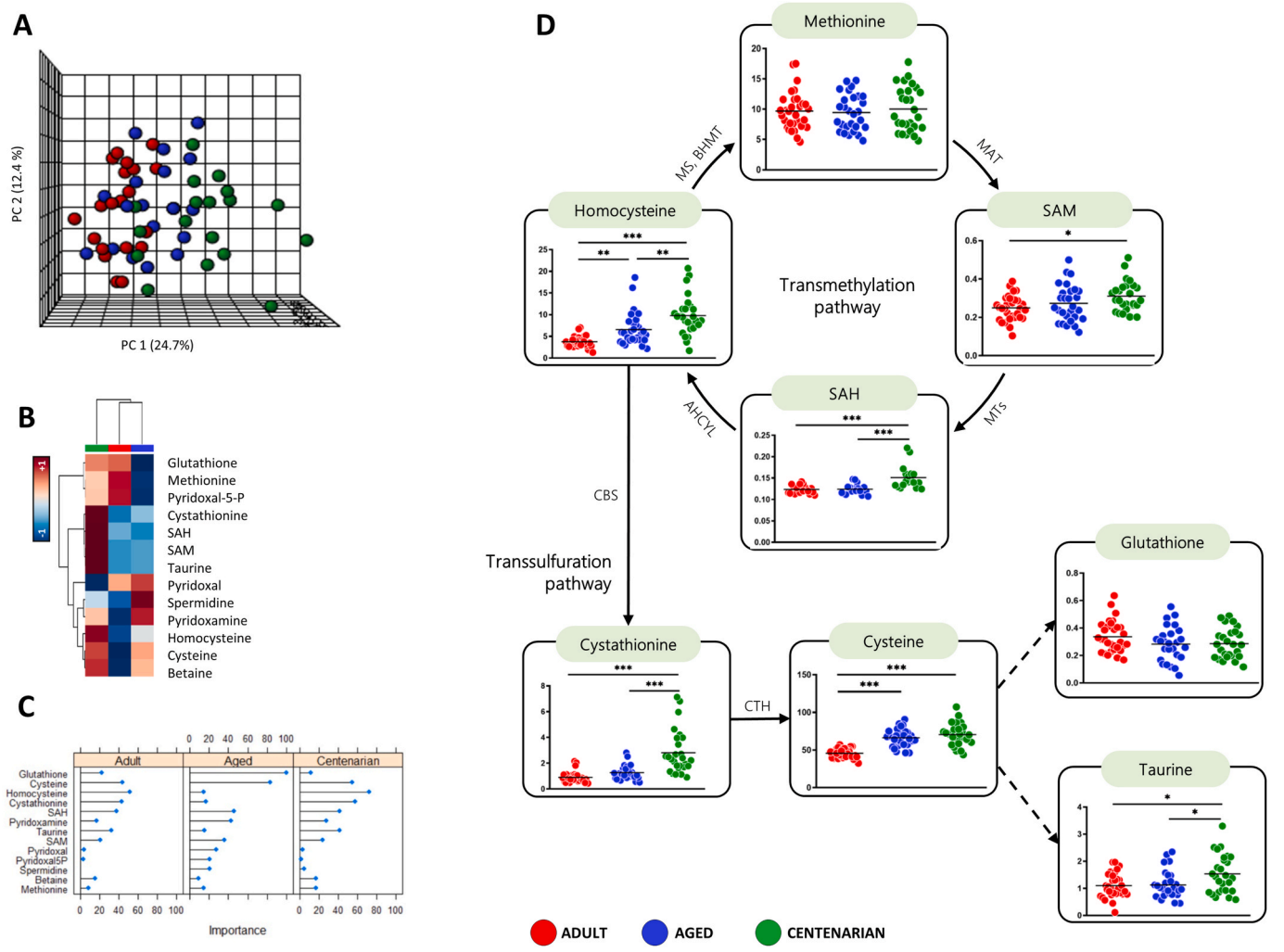

Fig. 2. Multivariate statistics reveals a centenarian-specific plasma methionine related metabolites profile. A) Principal component analyses (PCA) representation metabolite content. X: Principal component 1 (PC1); Y: Principal component 2 (PC2); Z (not shown): Principal component 3 (PC3), 11.6% (not shown). B) Hierarchical clustering of adult, aged and centenarian individuals according to average sample values of metabolite content. C) Scaled variable importance of Partial Least Squares Discriminant Analysis (PLS-DA) used for sample classification into each group.D) Individual metabolite plasma concentrations reported in μM. Dashed lines refer to reactions in which more than one enzyme is involved. Inter-group differences were measured by one-way ANOVA followed by a post-hoc Tukey multiple test. Minimum signification level was set at $p < 0.05$. Enzyme codes refer to: Methionine synthase (MS); Betaine-Homocysteine S-methyltransferase (BHMT); Methyltransferases (MTs); Cystathionine-β-synthase (CBS); Cystathionine-γ-lyase (CTH).

remaining 40%, obtaining an accuracy of 0.73 in the classification of the test data (binomial test, p[accuracy > no information ratio] = 0.0005). Permutation tests (1000 repeats) yielded a low $p < 0.001$, indicating that none of the distributions formed by the permutated data was better than the observed statistics based on the original data. The discriminating power between groups of the different measured features was ranked by applying the scaled variable importance score (Fig. 2C), indicating that glutathione, cysteine, homocysteine and cystathionine were the metabolites with higher weight discriminating groups. Specifically, glutathione and cysteine are the metabolites with a highest influence in aged group whereas cysteine, homocysteine and cystathionine defined better adult and centenarians.

2.2. Transsulfuration intermediates are increased in plasma from centenarians

The specific changes in methionine and its related metabolites content in plasma from centenarians were evaluated. Globally, methylation and transsulfuration metabolites were increased in plasma from centenarians (Fig. 2D, Supplementary Table 1). Specifically, centenarians have an increased content of SAH, homocysteine, cystathionine and taurine in comparison to adult and aged individuals. SAM and cysteine were also higher in centenarians in comparison with adults. Aged individuals were found to have higher levels of homocysteine and cysteine in comparison to adults. However, homocysteine levels in aged were still lower than those in centenarians. Adults were found to have the lowest plasma levels of homocysteine and cysteine. Methionine and glutathione plasma content remained unchanged among the different age groups, as well as betaine, spermidine and the vitamin B6 intermediate metabolites (Supplementary Fig. 1). Surprisingly, although glutathione presented no significant differences between groups, it had a high impact when defining aged group in multivariate statistics.

2.3. Centenarians and aged individuals maintain a similar amino acids plasma profile

Since methionine is a proteinogenic amino acid, we hypothesized the possibility that other amino acids could be also involved in the achievement of centenarian condition. Specifically, we have been able to unambiguously detect 14 additional amino acids apart from cysteine

and methionine. In order to determine whether plasma amino acids content differed among adult, aged and centenarian individuals, multivariate statistics were applied using the concentration of 16 plasma amino acids. Non-supervised PCA revealed the existence of an adults-specific plasma amino acids profile (Fig. 3A), capable to explain 42% of sample variability. A hierarchical clustering of the samples represented by a heat map revealed that centenarians maintain an aged plasma amino acid profile, and that methionine is associated with longevity (similar content between adult and centenarians) (Fig. 3B). These results were confirmed by performing a supervised analysis, such as PLS-DA, which showed that plasma metabolite content is a good model to identify the different groups. The model was trained using 60% of the samples and tested using the remaining 40%, obtaining an accuracy of 0.64 in the classification of the test data (binomial test, p[accuracy > no information ratio] = 0.008). Permutation tests (1000 repeats) yielded a low $p < 0.001$, indicating that none of the distributions formed by the permutated data was better than the observed statistics based on the original data. The discriminating power between groups of the different measured features was ranked by applying the scaled variable importance score (Fig. 3C). The results showed that homocysteine and cysteine were the two amino acids with highest weight defining plasma amino acid profile of adult and centenarians, followed by threonine and serine, whereas homocysteine and tyrosine had highest power in defining aged group, followed by glutamate and arginine. In order to discard that the metabolic signature was mainly determined by methionine metabolism metabolites, multivariate statistics were also performed without including the methionine-related amino acids (methionine, homocysteine, cysteine) and similar results were obtained (data not shown).

The specific changes plasma amino acids in centenarians were also evaluated (Fig. 3D–G, Supplementary Table 1). Among the 14 additionally detected amino acids (methionine and cysteine amino acids not included), we have found a specific decrease of tryptophan plasma content in centenarians. Serine, threonine and valine were also decreased in centenarians in comparison to adults. Asparagine, in turn, was increased in adults in comparison to aged and centenarians.

2.4. *Energy metabolism intermediates are increased in plasma from centenarians*

Considering that amino acids can be metabolized into TCA cycle intermediates, we have analysed the plasma changes for TCA cycle metabolites. Specifically, we had been able to unambiguously detect pyruvate and 6 TCA cycle intermediates, including citrate, isocitrate, α-ketoglutarate, succinate, fumarate and malate. In order to determine whether centenarians have a specific plasma profile for TCA cycle intermediates, multivariate statistics were applied using the plasma concentration of the mentioned metabolites. A hierarchical clustering of the samples represented by a heat map revealed that centenarians have a unique plasma TCA cycle intermediates profile (Fig. 4A), in terms of maintaining increased intermediates plasma content. Furthermore, we have identified a group of aged individuals that clustered together with some centenarians (Fig. 4B), which might correspond to aged individuals with an optimized energy metabolism and capable to reach the centenarian condition. However, multivariate statistics revealed that TCA metabolites did not discriminate centenarians group. PCA showed

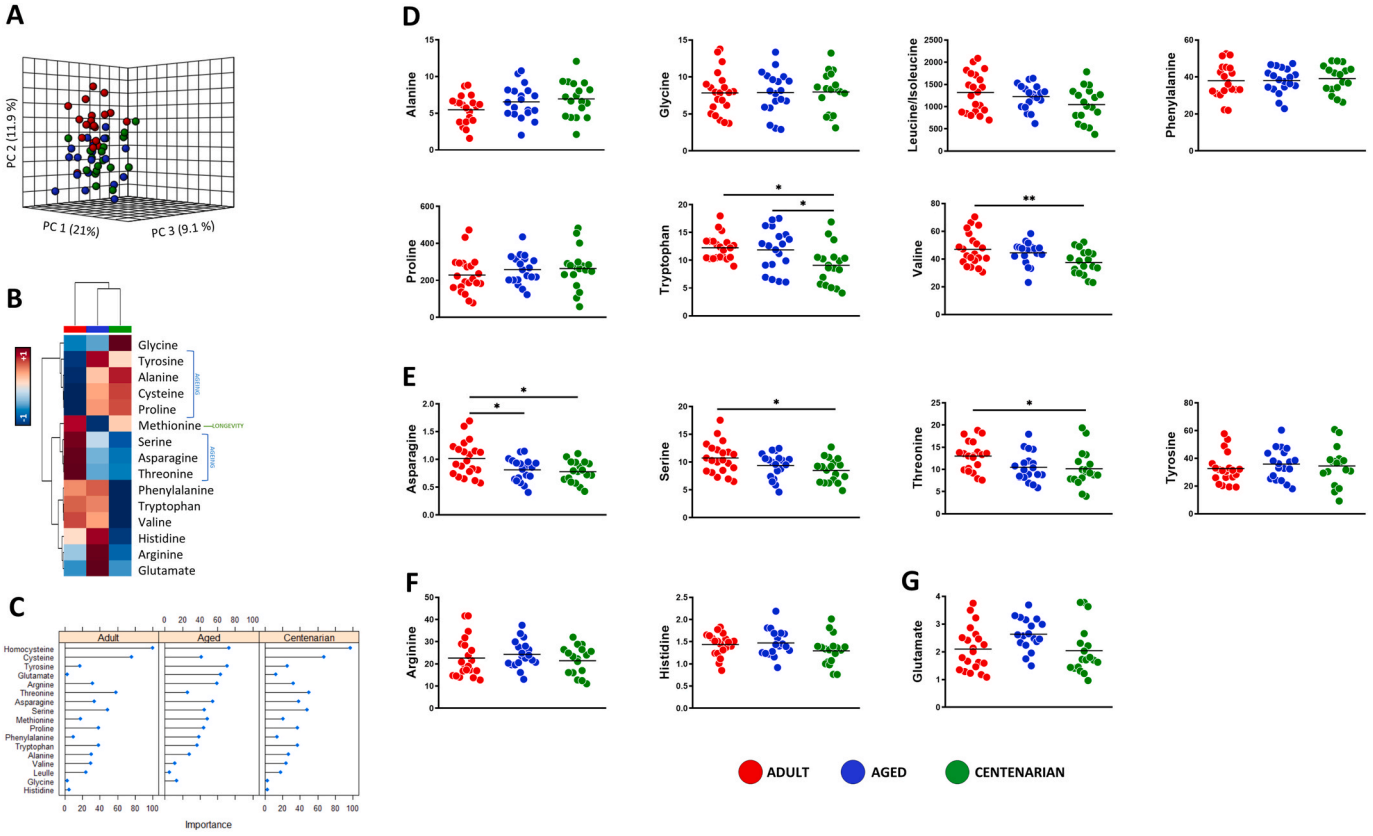

Fig. 3. Multivariate statistics reveals an adults-specific plasma amino acid profile. A) Principal component analyses (PCA) representation metabolite content. X: Principal component 1 (PC1); Y: Principal component 2 (PC2); Z: Principal component 3 (PC3). **B)** Hierarchical clustering of adult, aged and centenarian individuals according to average sample values of metabolite content. **C)** Scaled variable importance of Partial Least Squares Discriminant Analysis (PLS-DA) used for sample classification into each group. **D-G)** Individual plasma concentration reported in μM of non-polar (**D**), polar uncharged (**E**), negatively charged (**F**) and positively charged (**G**) amino acids. Plasma concentrations for leucine/isoleucine is reported in MS Counts. Inter-group differences were measured by one-way ANOVA followed by a post-hoc Tukey multiple test. Minimum signification level was set at $p < 0.05$.

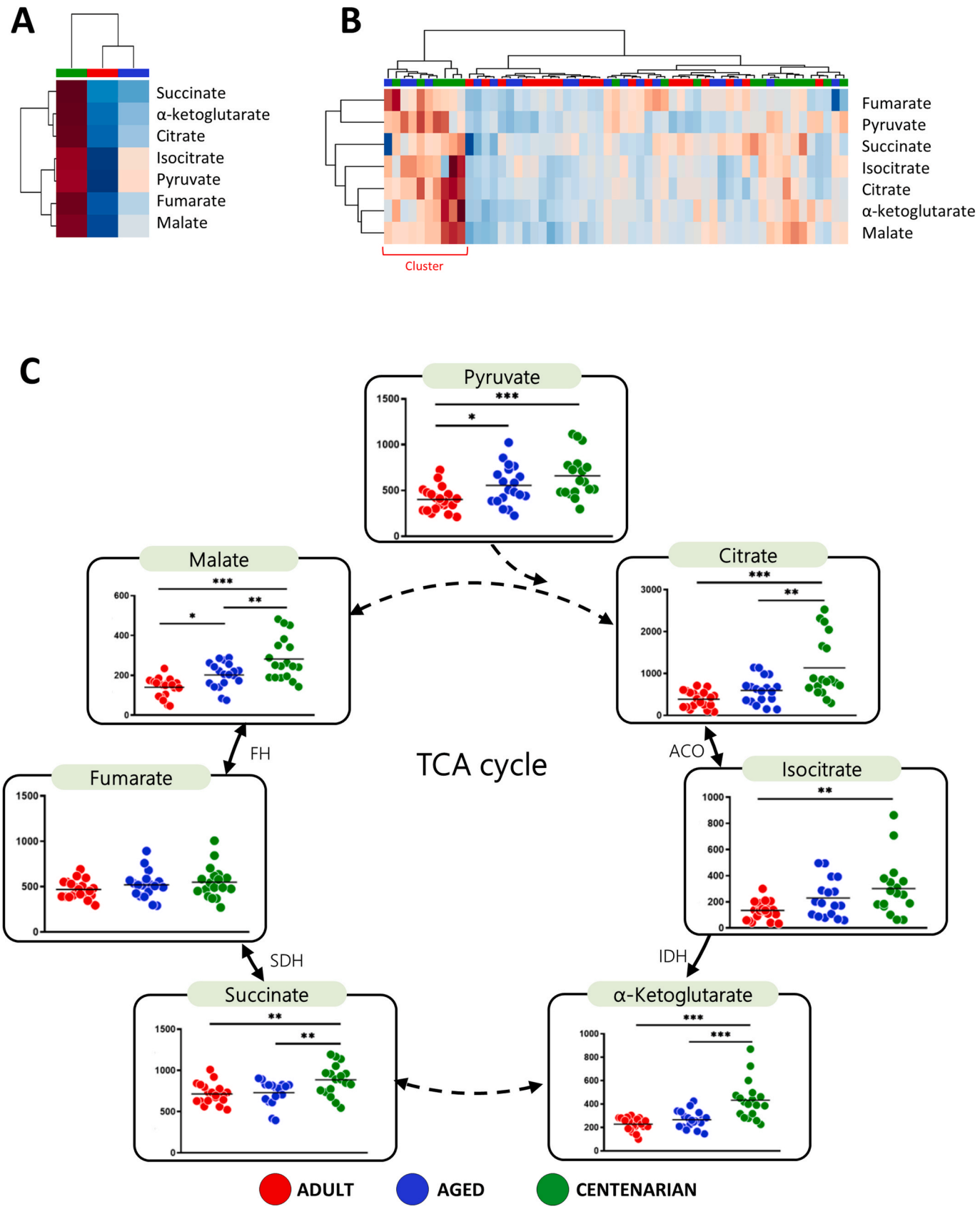

Fig. 4. Multivariate statistics reveals a centenarian-specific energetic metabolism plasma profile. A) Hierarchical clustering of adult, aged and centenarian individuals according to average sample values of metabolite content. B) Hierarchical clustering of individual samples according to metabolite content. C) Individual metabolite plasma concentrations reported in MS Counts. Dashed lines refer to reactions in which more than one enzyme is involved. Inter-group differences were measured by one-way ANOVA followed by a post-hoc Tukey multiple test. Minimum signification level was set at $p < 0.05$. Enzyme codes refer to: Aconitase (ACO); Isocitrate dehydrogenase (IDH or Cx I); Succinate dehydrogenase (SDH or Cx II); Fumarate hydratase (FH).

no differences between groups (Supplementary Fig. 2A) whereas PLS-DA accuracy for this model was 0.5 and the classification of the test data (binomial test, p[accuracy > no information ratio] = 0.16). The discriminating power between groups of the different measured features was ranked by applying the scaled variable importance score where succinate, pyruvate and fumarate arose as the TCA metabolites with highest weight defining aged condition (Supplementary Fig. 2B)

The specific changes in plasma intermediates of TCA cycle were also evaluated (Fig. 4C, Supplementary Table 1). Among the 7 detected metabolites, centenarians were found to have the highest plasma content of citrate, α-ketoglutarate, succinate and malate in comparison to adult and aged individuals. Pyruvate and isocitrate were also higher in centenarians in comparison to adults. Aged individuals were found to have higher pyruvate and malate levels, although plasma levels of malate were still lower that those found in centenarians. The lowest plasma content of pyruvate and malate were found in adults. Fumarate plasma content remained unchanged.

Amino acids and TCA cycle intermediates are bidirectionally related: the amino acids carbon skeleton can be used to synthetize TCA cycle intermediates and vice versa (Fig. 5A). Since plasma amino acids were decreased in centenarians, we have estimated the conversion of specific amino acids into the measured TCA intermediates (Fig. 5B). In fact, centenarians were found to have increased synthesis of pyruvate from serine and tryptophan, and synthesis of α-ketoglutarate from arginine, glutamate, histidine and proline. The synthesis of pyruvate from alanine, glycine and threonine was higher in centenarians in comparison with adults. Synthesis of pyruvate from serine and threonine was decreased in adults. In addition, positive correlations between pyruvate and serine and alanine were found in centenarians, and between pyruvate and alanine and threonine in adults (Supplementary Fig. 3).

2.5. *Centenarians plasma metabolome is also related with specific lipid intermediates*

Methionine metabolism participates in the biosynthesis of lipid intermediates such as choline and carnitine. Plasma choline levels were higher in centenarians than adults (Fig. 5C). Carnitine plasma levels, in turn, were increased in aged (Fig. 5C).

2.6. *Centenarians maintain network integrity*

In addition to identifying changes on individual metabolites content, we have also sought the underlying longevity mechanisms by identifying the metabolomic network integrity through correlation patterns within the different groups of the two main metabolic pathways analysed, methionine metabolism and TCA cycle.

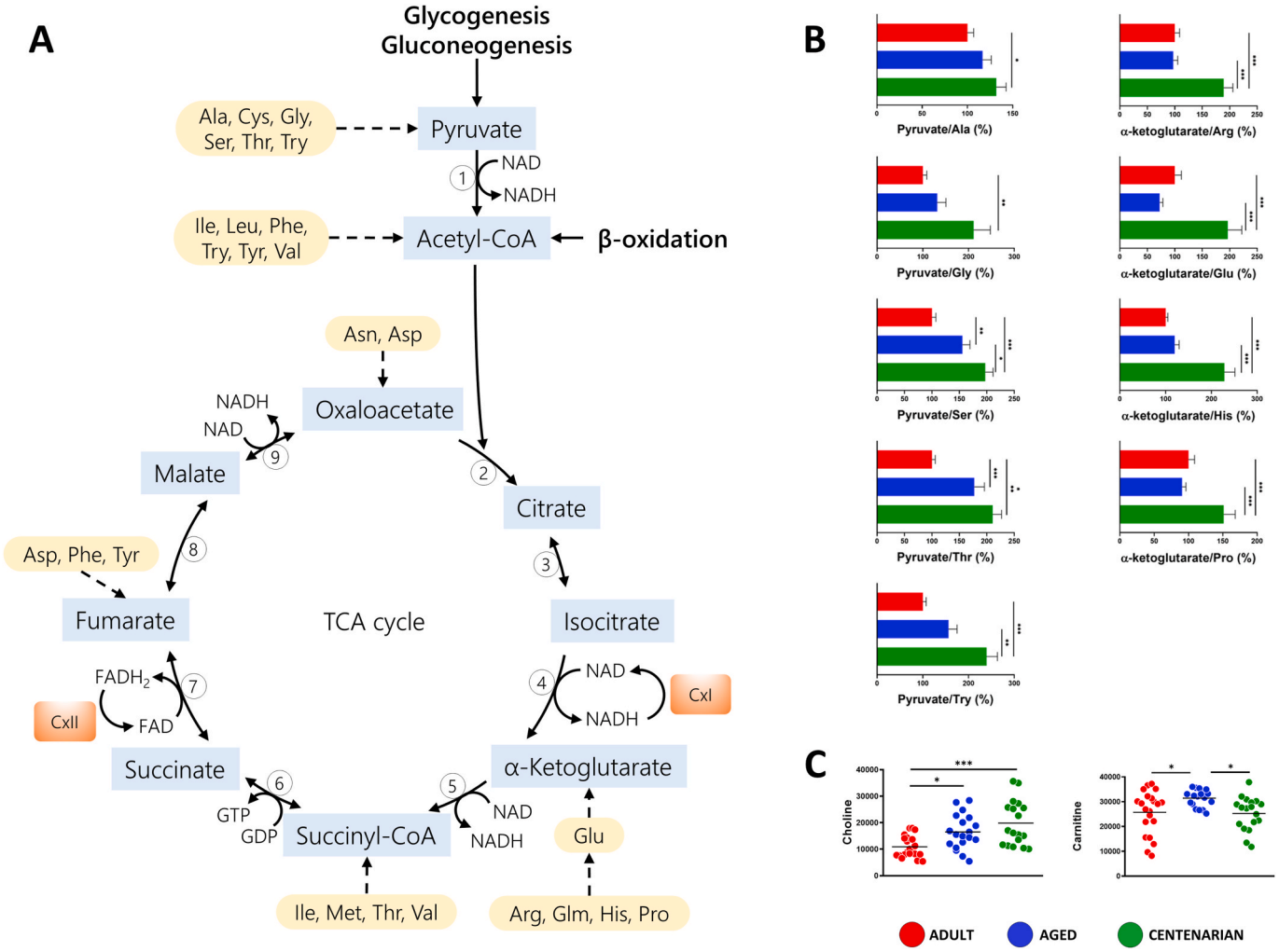

Fig. 5. Estimated conversion rate of amino acids into TCA cycle intermediates is enhanced in centenarians. A) Metabolic associations between amino acids and TCA cycle intermediates. Numbers refer to enzymes: 1, Pyruvate dehydrogenase (PDH); 2, Citrate synthase (CS); 3, Aconitase (ACO); 4, Isocitrate dehydrogenase or Cx I (IDH); 5, a-ketoglutarate dehydrogenase (OGDH); 6, Succinyl-CoA synthetase (SCS); 7; succinate dehydrogenase or Cx II (SDH); 8, Fumarate hydratase (FH); 9, Malate dehydrogenase (MDH). B) Estimated conversion rate of amino acids into TCA cycle intermediates. C) Lipid intermediates plasma concentration. Inter-group differences were measured by one-way ANOVA followed by a post-hoc Tukey multiple test. Minimum signification level was set at $p < 0.05$.

The network plot revealed different hubs (metabolites with multiple interactions) and signalling network for methionine metabolism in plasma for each group (Fig. 6A–F). Accordingly, centenarians maintain a unique signalling network being cysteine the central hub (Fig. 6A–C), with a stronger correlation degree in comparison to adult and aged individuals (Supplementary Fig. 4). In adults, we have found an ordered pattern, characterized by correlations within the transmethylation and transsulfuration metabolites separately (Fig. 6D). Specifically, SAM was negatively correlated with betaine and choline. The sulphur-containing metabolite cystathionine was positively correlated with both homocysteine and pyridoxamine. In aged individuals, no correlations between the transmethylation metabolites were found (Fig. 6E). Regarding sulphur-containing metabolites, cysteine was positively correlated with cystathionine and glutathione. Pyridoxamine was also positively correlated with glutathione. In both groups, positive correlations between vitamin B12 intermediates pyridoxal and pyridoxamine were also found. Centenarians, in turn, show a complex but ordered correlation pattern (Fig. 6F). Accordingly, of biological significance are the correlations found within i) the trans-methylation metabolites (positive correlation of SAM with methionine and SAM); ii) the transsulfuration metabolites (positive correlation of cystathionine with homocysteine, cysteine and pyridoxamine); and iii) sulphur-containing metabolites (positive correlation between cysteine and taurine).

TCA cycle network plot also revealed different plasma signalling network for each group (Fig. 6G-L). Surprisingly, the highest (Fig. 6G–I) and strongest (Supplementary Fig. 4) correlation degree was found in aged individuals, which show a disordered signalling network. Positive correlations between fumarate and malate, malate and citrate, and citrate and isocitrate, were found in adults, suggesting the existence of a continuous flux of reactions from fumarate to isocitrate (Fig. 6J). In aged individuals, citrate was positively correlated with malate, pyruvate and isocitrate (Fig. 6K). Positive correlation between fumarate and isocitrate was also found. However, this correlation pattern revealed a fragmented network, with a flux of reactions from malate to isocitrate, depending upon pyruvate, and isocitrate to fumarate. Centenarians, in turn, show positive correlations between malate and citrate, citrate and isocitrate, and isocitrate and α-ketoglutarate were also found, suggesting in this case, a continuous flux of reactions from malate to α-ketoglutarate (Figure 6L).

3. Discussion

3.1. Transsulfuration is enhanced in centenarians and leads to a unique methionine plasma profile

Human longevity benefits from an organismal reorganization of its whole metabolism. Several intracellular signalling pathways are modulated, but mostly converge in the modulation of a small set of pro-longevity genes [19]. Consequently, this leads to an specific gene expression pattern [20–23] which supports specific proteomics [24], metabolomics [11,19,25] and lipidomics [26,27] profile associated with human longevity. In our study, we have identified that methionine plasma metabolome is associated with exceptional human longevity. Our model revealed that using trans-methylation and transsulfuration metabolites we are able to explain the centenarian condition, being the transsulfuration metabolites cysteine, homocysteine and cystathionine the highest longevity predictors.

The association between methionine and longevity has been deeply studied. Long lived mammals have lower tissue [3–5] and plasma [11, 28] content of methionine, and methionine restriction (MetR) leads to an extended longevity in different experimental models [9,29–32]. Methionine is an essential amino acid that is involved in several intracellular processes (Fig. 1), such as intracellular metabolism and signalling, methylation reactions, maintenance of redox balance, protein synthesis, autophagy, and biosynthesis of polyamines or nucleotides [15]. Likely due to its versatility, and the involvement of these intracellular processes with the determination of animal longevity, we have unfocused solely from methionine and analysed the changes on its related metabolites. Our results revealed that centenarians have higher levels of homocysteine, cystathionine, cysteine and taurine, suggesting an increased transsulfuration directed to increase the plasma levels of taurine, without changing methionine plasma content.

The observed metabolic adaptations associated to human longevity regarding methionine metabolism reveal new insights on inter-individual longevity. It has been previously discussed the existence of factors exerting a “big effect” on longevity determinations, along with factors inducing a “small effect” on longevity [33]. Inter-species studies are a powerful source of information to identify mechanism inducing a “big effect” on extended longevity, such as maintaining lower methionine content. These mechanisms determine whether an animal species lives longer than others. However, these mechanisms usually differ from the specific individual metabolic adaptations determining whether a specific individual of a species lives longer than the average, and have a “small effect” on longevity determination. Our study aims to determine the metabolic adaptations leading to human longevity, and the obtained results suggest that although the steady states of plasma methionine decrease with animal longevity, the specific modulations on methionine metabolism, mainly at the transsulfuration pathway, and not methionine content *per se*, determines whether a specimen/individual from one species lives longer than its life expectancy, reaching a longevity condition. In fact, it is well-established that MetR in rodents induces hyperhomocysteinemia [29–32], probably due to an enhanced transsulfuration, as suggested previously [34]. In a recently published study, men that underwent 45 min of cycling for 12 weeks showed decreased plasma methionine and increased plasma concentrations of homocysteine, cystathionine, cysteine, glutathione and taurine [35], supporting the beneficial effects of an enhanced transsulfuration.

Transsulfuration starts with the metabolic conversion of homocysteine into cystathionine, an enzymatic reaction catalyzed by the enzyme CBS, using vitamin B6 as cofactor. According to our hypothesis, we have found that cystathionine is significantly increased in centenarians. Reinforcing this finding, SAM, an allosteric activator of CBS [36], is increased in centenarians and positively correlated with cystathionine. In fact, it has been demonstrated in rodents that injected isotope-labelled methionine is mainly metabolized via the transsulfuration pathway in long living Ames dwarf mouse, along with an enhanced gene expression of methionine adenosyltransferase (MAT), methyltransferases (MTs), betaine-homocysteine methyltransferases (BHMT), adenosylhomocysteinase-like1 (AHCYL) and CBS, but decreased methionine synthase (MS) [37]. Furthermore, dietary restriction increases longevity in flies by promoting transsulfuration and CBS activity [38].

Proteins are in continuous turnover and exchanges with the free amino acid pool [39], such as free methionine, which is essential for the initiation of protein synthesis. Interestingly, methionine plasma levels remained unchanged across the different groups, likely due to its strong regulation. Supporting this idea, plasma methionine levels also remain stable in ageing mice [40]. The maintenance of free methionine mainly depends on the transmethylation and the methionine salvage pathway. The transmethylations involves either MS, which requires 5′-methyltetrahydrofolate as a methyl donor; BHMT, which requires betaine as a methyl donor; or the methionine salvage pathway that produces spermidine. Betaine and spermidine plasma levels remained unchanged, and methionine salvage pathway was decreased in centenarians. According to this data, we suggest that although transsulfuration is enhanced, centenarians maintain decreased but sufficient MS activity to maintain methionine plasma levels.

Methylation is needed to modulate gene expression, enzymatic activity and to promote the biosynthesis of several metabolites. It relies upon methyltransferases (MTs), which are the enzymes responsible to donate a methyl group from SAM to DNA, RNA, proteins and lipids, generating SAH. In our model, methylation is associated with human

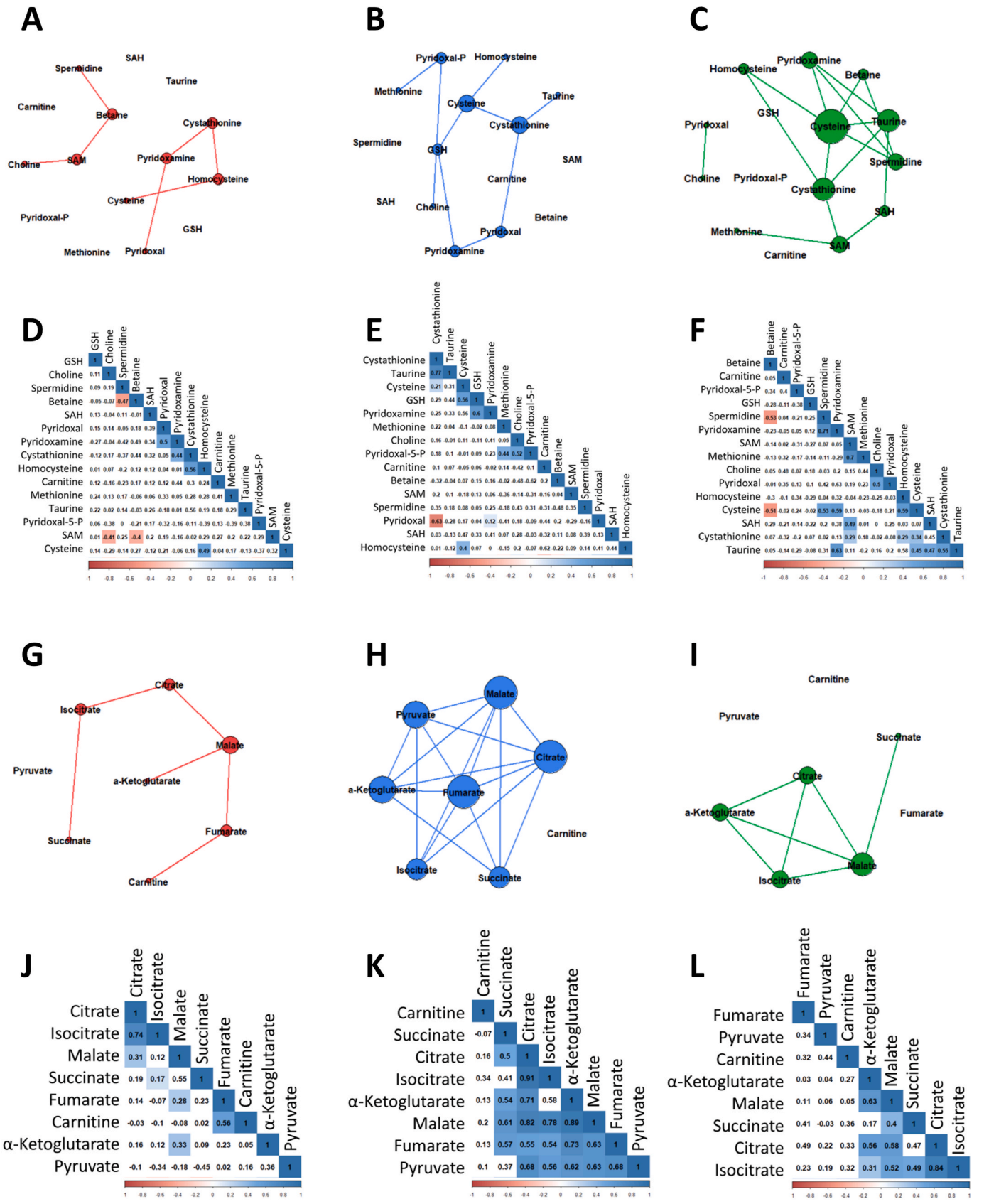

Fig. 6. Network integrity of methionine metabolism and TCA cycle in adult, aged and centenarian individuals. Network plot revealing significant and strong correlations ($r > 0.65$) between methionine metabolites in adults (A), aged (B) and centenarian (C) individuals. Pearson correlation matrix of methionine metabolites in adult (D), aged (E) and centenarian (F) individuals. Network plot revealing significant and strong correlations ($r > 0.65$) between TCA cycle intermediates in adults (G), aged (H) and centenarian (I) individuals. Pearson correlation matrix of TCA cycle intermediates in adult (J), aged (K) and centenarian (L) individuals. Dot size depends upon hubs.

extreme longevity, since plasma content of SAM and SAH is increased. Methylation has been previously positively associated with longevity [14,41–44]. Accordingly, MAT activity was higher in liver of Snell dwarf, and the increased SAM was positively correlated with DNA methylation status [34]. PEMT is a methyltransferase that catalyses de biosynthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE). The resulting PC can be then hydrolyzed via the action of phospholipases to generate choline, constituting the only known endogenous pathway for choline biosynthesis in mammals [45]. Choline plasma levels are increased in centenarians, supporting the modulation of lipid metabolism associated to human longevity. Interestingly, liver PEMT activity is inhibited in Alzheimer's disease patients [46].

Globally, the obtained results suggest that centenarians undergo a specific remodulation of the methionine metabolism, being an enhanced transsulfuration and transmethylation two key traits (Fig. 7). Furthermore, we believe that these two processes are directed to increase the plasma levels of taurine and to modulate the lipidomic profile. Taurine is a cytoprotective β-amino acid with antioxidant properties that has been described to modulate pro-longevity genes [47] and its intake is associated with human longevity and cardiovascular health [48]. Furthermore, taurine plasma levels are higher in long-lived Dwarf mice than in control mice [34]. Adults, in turn, promote the transmethylation and the synthesis of glutathione, an antioxidant compound. In both situations, increased plasma levels of homocysteine, which has been described as a cardiovascular risk factor [49], are cleared. Contrarily, the increased homocysteine in aged individuals accumulates, due to the disruption of the transmethylation and transsulfuration metabolism, leading to the cardiovascular comorbidities associated to the ageing process.

3.2. *Decreased amino acid content in centenarians*

Amino acids constitute the building blocks for proteins, the functional biomolecules in our body. However, they can also be found as free metabolites, functioning as signalling molecules or metabolic intermediates. The findings associating methionine metabolism intermediates and human longevity, as well as the strong metabolic interconnection between amino acids, led us to a challenging question: Is the whole amino acid metabolic profile modulated in longevous individuals? And if it's so, does this modulation affect all the amino acids in the same way, or it occurs through specific and individualized amino acid changes?

Our data revealed that although some amino acid levels are modulated by the ageing process, methionine remains unchanged between adult and centenarians, and plasma cysteine constitutes the most important feature to discern between groups. As previously discussed, this probably might be due to the relevance of the transsulfuration pathway in longevity determination. This centenarian profile is characterized by decreased tryptophan, but also serine, threonine, and

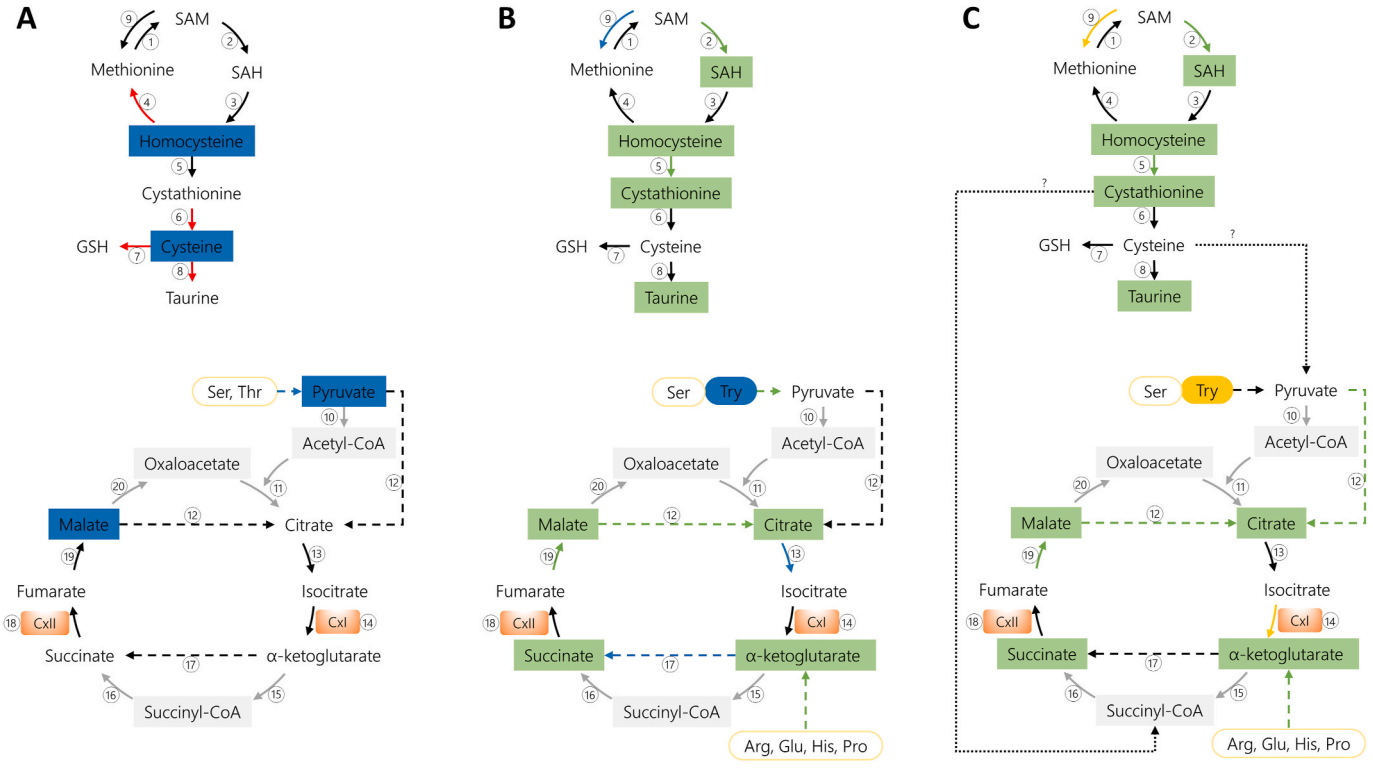

Fig. 7. Proposed model for methionine and energetic metabolism plasma profile associated to human longevity. A) Plasma metabolome changes associated to human ageing (*adult vs aged individuals)*. B) Differential plasma metabolome traits associated to ageing and longevity (*aged vs centenarian individuals*). C) Plasma metabolome associated to human longevity (*centenarian vs adult and aged individuals*). Red shaded arrows refer to reactions enhanced in adults. Blue shaded boxes or arrows refer to metabolites or reactions enhanced in aged. Green shaded boxes or arrows refer to metabolites or reactions enhanced in centenarians. Yellow shaded boxes or arrows refer to metabolites or reactions specifically diminished in centenarians (*centenarians vs adult and aged individuals*). Grey shaded boxes or arrows refer to non-detected metabolites or not-estimated reactions. Solid lines refer to enzymatic reactions in which one enzyme is involved. Dashed lines refer to enzymatic reactions in which more than one enzyme is involved (enzyme bypass according to data availability). Dotted black lines refer to hypotheses linking methionine and energetic metabolism. Numbers refer to enzymes or ratios: 1, Methionine adenosyltansferase Iα/IIα (MAT1A/MAT2A); 2, Methyltransferases (MTs); 3, Adenosylhomocysteinase-like 1 (AHCYL); 4, Methionine synthase (MS); 5, Cystathionine-β-synthase (CBS); 6, Cystathionine-γ-lyase (CTH); 7, Glutathione biosynthesis (ratio glutathione/cysteine); 8, Taurine biosynthesis (ratio taurine/cysteine); 9, Methionine salvage pathway (ratio (spermidine + met)/SAM); 10, Pyruvate dehydrogenase (PDH); 11, Citrate synthase (CS); 12, Citrate biosynthesis from malate and pyiruvate (ratio citrate/(malate + pyruvate)); 13, Aconitase (ACO); 14, Isocitrate dehydrogenase or Cx I (IDH); 15, a-ketoglutarate dehydrogenase (OGDH); 16, Succinyl-CoA synthetase (SCS); 17, Ratio succinate/a-ketoglutarate; 18; succinate dehydrogenase or Cx II (SDH); 19, Fumarate hydratase (FH); 20, Malate dehydrogenase (MDH). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

valine. It has been reported that tryptophan is needed for the *de novo* NAD^+ synthesis, which enhances mitochondrial function and improves health by conserved mechanisms from invertebrates to mammals [50]. Furthermore, a recent study [51] demonstrated a promoting aging role for serine, threonine, and valine through the specific activation of Ras/cAMP/PKA, PKH1/2 and Tor/S6K pathways. Consequently, the low content of these amino acids detected in centenarians must be interpreted as a pro-longevity factor by down-regulating the mentioned pathways.

The progressive loss of muscle mass and strength, referred to as sarcopenia, occurs during the ageing process [52]. Metabolically, this might be expressed as an enhanced catabolism of proteins and thus increased free amino acid pool. Accordingly, it has been suggested that the intake of essential amino acids prevents this process in older women [53]. Therefore, the reduced plasma content of selected amino acids in centenarians might also be an indicative of a proper maintenance of muscle mass in comparison to aged individuals.

Besides, previous studies in longevity models had already described a global decrease of peptides and amino acids in tissue and plasma [54,55], along with an increased proteasomal activity and autophagy [11]. Furthermore, nutritional interventions in flies [56] and humans [57] also led to decreased tissue and plasma amino acid content, although some studies associate longevity with increased amino acids [58,59].

3.3. Energetic metabolism is enhanced in centenarians

Mitochondria has been pointed as a central hub in longevity determination. Decreased reactive oxygen species production [10], quantitative and qualitative modulations of the electron transport chain complex I [10,12,60], decreased membrane unsaturation [10] and lower permeability [12,61], and modulation of mitochondrial dynamics [62] represent, among others, structural and functional adaptations leading to a mitochondrial phenotype associated to organismal longevity. The intracellular role of the mitochondria is to provide the cell with ATP obtained either from oxidation of carbohydrate, proteins and fats, although it is also involved in gluconeogenesis, amino acids and one-carbon metabolism, and lipid [63] and protein synthesis [64]. Due to the previously mentioned structural and functional adaptations, its highly feasible to postulate that mitochondrial metabolism may be also modulated in long-lived individuals.

The TCA cycle occurs in the mitochondrial matrix and constitutes a metabolic epicentre because multiple substrates can feed into it [65]. The TCA begins with two molecules of acetyl-CoA, generated from fatty acids, amino acids or pyruvate oxidation, that are subsequentially metabolized into different intermediates. During these reactions, GTP and electron donors are generated (e.g., NADH and FADH), that will transfer those electrons to the electron transport chain transporters to subsequentially generate ATP. In our study, we have found that centenarians have a global plasma increase of the TCA cycle intermediates, including citrate, α-ketoglutarate, succinate and malate, suggesting an optimized energetic metabolism. These results are supported by data across experimental models. Gene expression and activity of TCA cycle is enhanced in long-lived yeast mutants [66,67]. In long-lived rodents, TCA cycle genes are also upregulated [68], and its function is preserved with ageing [69]. Data in humans is scant, and revealed higher levels of malate in octogenarians, which were associated with higher cardiovascular risk [25]. Accordingly, higher levels of malate were found in aged in comparison with adults, although these levels were still higher in centenarians.

Pyruvate is versatile metabolite generated from glycolysis that can be used to synthetize carbohydrates (gluconeogenesis), acetyl-CoA, oxaloacetate (a TCA cycle intermediate), alanine or lactate. It can enter into the first step of the TCA cycle via its decarboxylation to acetyl-CoA, or its carboxylation to oxaloacetate. Pyruvate plasma levels are increased in centenarians, suggesting a proper glycolysis and TCA cycle activity. The beneficial effects of pyruvate supplementation had been previously reported, including oxidative stress protection [70] and lifespan extension via HIF-1 stabilization [71] in *C. elegans*, as well as increased explorative activity in mice Alzheimer disease models [72].

Citrate is the first TCA cycle intermediate, and its biosynthesis from acetyl-CoA constitute a limiting reaction of the metabolic pathway. In centenarians, citrate plasma levels are increased, probably due to its enhanced biosynthesis and the decreased estimated aconitase activity, which metabolizes citrate into isocitrate. Supporting this data, serum content of citrate had been previously reported to be higher in centenarians compared to aged individuals [59]. Decreased aconitase is associated with higher odds of living longer than 80 years without cardiovascular diseases [25]. When glucose is limited, citrate can be transported to the cytoplasm via the citrate transport protein (CTP), which exchanges mitochondrial citrate for cytosolic malate [73]. In the cytoplasm, citrate can be broken down into acetyl-CoA and OAA by the ATP citrate lyase (ACLY). The produced acetyl-CoA can be either used for *de novo* lipid biosynthesis or to acetylate proteins and DNA. Lower ACLY activity and histone methylation is associated with longevity in *D. melanogaster* [74]. However, genome-wide association studies (GWAS) in humans reported a significant association of citrate plasma content with SLC25A1, which encodes CTP [75]. Altogether these results suggest that retaining citrate in the mitochondria to maintain the TCA cycle is associated to longevity, and with increased plasma citrate, although the molecular mechanisms are unknown.

α-Ketoglutarate is synthetized by the oxidation and decarboxylation of isocitrate via isocitrate dehydrogenase (IDH) or complex I (Cx I). It constitutes a hub of anaplerotic reactions, since it can be synthetized from glutamate via glutamate dehydrogenase, allowing to maintain a proper metabolic activity and ATP generation. In fact, α-ketoglutarate is not only a metabolite, but also a cofactor of multiple dioxygenases that generate succinate as a by-product and are involved in the hypoxic response, histone methylation and inflammation, among others [65,76]. The activity of these α-ketoglutarate-dependent dioxygenases is inhibited by a high ratio succinate/α-ketoglutarate or fumarate [65]. Elevated α-ketoglutarate plasma levels were found in centenarians. According to literature, maintaining elevated α-ketoglutarate would ensure a proper ATP generation, as well as to promote the activity of the α-ketoglutarate-dependent dioxygenases [65]. Recently it has been associated with longevity, since α-ketoglutarate supplementation increases *C. elegans* longevity by decreasing ATP synthesis and inhibiting TOR [77].

Succinate is the substrate of the enzyme succinate dehydrogenase (SDH) or complex II (Cx II), thus its availability is essential for the production of ATP via the mitochondrial electron transport chain. Up to date, it is the only TCA cycle intermediate known to trigger organismal functions, by modulating renin-angiotensin system through its receptors SUCNR1, regulating the immune system and inducing thermogenesis in brown adipocytes [65]. Succinate plasma levels are increased in centenarians, suggesting a proper maintenance of ATP supply, as well as to maintain a proper immunity. Accordingly, increased succinate was found in worm long-lived models [58] and in response to MetR in flies [78].

Malate plasma levels were also increased in centenarians. It has been previously suggested that succinate increases due to fumarate accumulation lead to alterations in mitochondrial function [79–82]. Therefore, it seems that high plasma levels of malate might be an indicator of globally proper mitochondrial function and fumarate clearance.

In some situations, amino acids can be used to synthetize other metabolites, such as glucose or ketone bodies. Specifically, alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, methionine, proline, serine and valine are named as glucogenic amino acids, whereas leucine and lysine are ketogenic. Isoleucine, phenylalanine, threonine, tryptophan and tyrosine can be both glucogenic and ketogenic. As it has been mentioned previously, TCA cycle can be feed by amino acids. In fact, the carbon skeleton of several TCA cycle intermediates can also be used to synthetize amino acids. Accordingly, the obtained results suggest that pyruvate and α-ketoglutarate increases

might be, in part, due to its synthesis from amino acids.

Available literature suggests that increased plasma profile of TCA cycle intermediates might be due to modulations on its membrane transporters. INDY (*I'm not dead yet*) is a membrane transporter showing a high affinity for citrate and succinate, but also for α-ketoglutarate and fumarate. Studies on animal models suggest that INDY may account, in part, for extended longevity, occurring with increased plasma levels of the mentioned metabolites [83]. INDY mutations in worms [84] and fly [85,86] extend lifespan, and are related to dietary restriction phenotypes in mice [87]. Consequently, it has raised as a pharmacologic target in longevity [83]. However, no studies in human measuring the activity of these specific transporters have been published yet.

Globally, the obtained results suggest that centenarians undergo a metabolic modulation targeted to enhance the TCA cycle activity (Fig. 7). Glucose metabolism, but also amino acid catabolism, constitute metabolic pathways that fuel the cycle, by maintaining elevated levels of pyruvate and α-ketoglutarate. Furthermore, human longevity is achieved through the maintenance of elevated plasma levels of citrate, α-ketoglutarate and succinate, which might allow a proper energy supply (probably through the Cx I and II of the electron transport chain), histone acetylation and modulation of specific gene expression profile, as well as proper inflammation and immune response. The achievement of individual longevity has been widely associated with enhanced autophagy [88–91]. Thus, we cannot discard the possibility that enhanced levels of TCA cycle intermediates might not only respond to signalling functions, but also reflect an enhanced mitophagy to clear old dysfunctional organelles and maintain proper mitochondrial, thus cellular, tissue and organismal function.

3.4. Network integrity

The interaction between metabolites in large networks, in addition to changes in individual metabolites content, is critical for metabolism function and a source for the generation of new hypothesis about longevity. Networks consist in a set of "nodes" (in this case, metabolites), that are connected through "edges", which are commonly referred as correlations. Therefore, two metabolites that aren't directly connected in a metabolic pathway may nonetheless be connected if their concentrations are correlated with each other [92]. Network connectivity constitutes a mean of measuring network integrity. Accordingly, loss of network connectivity and non-communicated metabolites leads to cellular homeostasis disruption, as well as mitochondrial dysfunction, genomic instability and proteostasis loss [93]. Our work revealed that centenarians maintain a strongly correlated methionine intermediates, suggesting an improved network integrity, homeostasis and more tightly regulated metabolism. It had been previously demonstrated that longevity is associated with specific correlation networks [54], which can be modulated through nutritional interventions such as dietary restriction (DR) [94]. Globally, lifespan extension during DR occurs along with the strengthening [56] or maintenance of network integrity [56,94,95], even when no changes in mean levels of metabolites are found [56]. However, for TCA cycle intermediates, the strongest correlation network was found in aged, showing a disordered correlation network. Conversely, it has been suggested that network connectivity often declines with age [96]. In this case, increasing or decreasing the correlation degree is not sufficient to define network integrity. Although the performance of correlation matrix provides new and useful information, it is also important to identify the biological significance of these correlations, and to combine this data with changes in the individual metabolites. Accordingly, we postulate that the higher and stronger correlation pattern found in aged individuals reflects the loss of homeostasis that occurs through the aging process, and the failed efforts of intracellular metabolism to restore it.

4. Conclusions

In the present work, we define a plasma profile associated to human longevity characterized by an enhanced transsulfuration and TCA cycle intermediates, as well as a reduced specific amino acid content (Fig. 7). Globally, this metabolic profile might suggest an enhanced energetic metabolism. The connection between transsulfuration and TCA cycle intermediates is through anaplerotic reactions, such as the conversion of cystathionine into succinyl-CoA, and cysteine into pyruvate. Accordingly, methionine supplementation enhanced mitochondrial pyruvate uptake and TCA cycle activity [97]. Furthermore, we suggest that enhanced TCA activity is promoted by the synthesis of its intermediates from amino acids. In this line, it must be highlighted a recent study [98] performing an untargeted metabolic analysis in tissue from mice feed with different diets (*ad libitum* and DR) and intake strategies. The authors reported that liver glycine-serine-threonine and taurine metabolism, and transmethylation and transsulfuration pathways were the top enriched pathways associated to longevity, independent of diet and feeding regimen. TCA cycle was also found to be modulated as a function of feeding behavior but independent of diet. Furthermore, they suggest that longevity is associated with a high degree of metabolic connectivity between antioxidant and energy signalling pathways, in which amino acids play a central role. The authors also evaluated the plasma composition associated to the different diets and food intake strategies in mice and non-human primates. Interestingly, they reported that amino acids are conserved factors associated to longevity not only in tissue but also in serum. Nonetheless, more work is needed to elucidate the organismal functions of the transsulfuration and TCA cycle metabolites and its affinity with its specific transporters, as well as the activity modulation or genetic variants of the enzymes involved in the methionine and energetic metabolism, along with tissue flux measurements in the context of human longevity.

However, our work has some limitations. First, the use of a targeted approach leads to intrinsically biased results. Although we have tried to be the most accurate when selecting the methionine-related metabolites, we cannot rule out the possibility that we had left out other related metabolites that could be as, or even more, important as the ones we had measured. Second, the limited number of measured metabolites leads to uncompleted networks. Although previous data allow us to generate new hypothesis, it should be needed to identify more metabolites in order to define a global network. Specifically, it would be interesting to include intermediate metabolites within the pathways, in order to establish clear associations between methionine metabolism, TCA cycle and amino acids. Third, we have measured a plasma profile, which is dynamic and undergoing continuous changes. It allows us to get a global view of the metabolic processes undergoing in the organisms, but it's also a source of metabolic precursors that are taken by cells to be metabolized. Furthermore, we should keep in mind that metabolic reactions occur within organs, not in plasma, and that metabolic profile is tissue-specific. Therefore, although we hypothesize that the obtained plasma profile might be a reflect of tissue metabolic status, this obtained plasma profile should be seek and confirmed in other organs. And fourth, the study is performed in a small population from a limited region in Spain. Therefore, the obtained results should be validated in a different and bigger cohort.

5. Methods

5.1. Chemicals

Unless otherwise specified, all reagents were from Sigma-Aldrich, and of the highest purity available.

5.2. Sample population

Potential healthy subjects were selected from the population data

system of the 11th Health Department of the Valencian Community (Valencia, Spain), which is composed of 29 towns (240,000 inhabitants). The inclusion criteria were to live in the 11th Health Department for at least the last 6 years and to sign the informed consent. The exclusion criterion was to be under statin-therapy or any pharmacological treatment affecting lipid metabolism or to be terminally ill for any reason. We included 18 centenarians (100.8 ± 1.1 years), 21 randomly recruited aged subjects (76.4 ± 0.5 years), and 21 adult individuals (27.9 ± 1.4 years). All experimental procedures were approved by the Committee for Ethics in Clinical Research of the Hospital de la Ribera (Alzira, Valencia, Spain). All subjects or their relatives were fully informed of the aims and scope of the research and signed an informed consent.

5.3. Blood collection and plasma isolation

Blood samples were obtained by venipunction in the morning (between 7 and 8 a.m.) after fasting overnight (8–10 h) and collected in one vacutainer CPT (Cell Preparation Tube; BD, Franklin Lakes, NJ) containing sodium heparin as the anticoagulant. Plasma fractions were collected after blood sample centrifugation, and immediately frozen in liquid nitrogen, and transferred before 4 h to a −80 °C freezer for storage, to be used later for metabolomic analyses.

5.4. Sample processing

Plasma metabolites extraction was performed based on the methodology previously described (Method 1 [99]. Briefly, 10 μL of plasma were added to 30 μL of cold methanol containing 1 μg/mL of Phe-^{13}C as internal standard and 1 μM BHT as antioxidant. Then, samples were incubated at room temperature for 15 min and centrifuged at 12,000 *g* for 3 min. Finally, the supernatant was filtrated through a 0.22-μm organic diameter filter (CLS8169, Sigma, Madrid, Spain) and were transferred to Agilent (Barcelona, Spain) vials with glass inserts for further analysis.

Sulphur-containing metabolites were extracted on the bases of the methodology previously described (Method 2 [100]). Briefly, 2 μL of 5% DTT diluted in methanol (m/v) were added to 10 μL of plasma. The resulting solution was vortexed for 1 min and allowed to stand at room temperature for 10 min. For protein precipitation, 40 μL of acetonitrile containing 0.1% formic acid (v/v), 0.05% trifluoroacetic acid (v/v) and 1 μg/mL of Phe-^{13}C as internal standard was added to the sample, and the solution was vortexed for 2 min. Then, samples were incubated at room temperature for 15 min and centrifuged at 12000 *g* for 3 min. Finally, the supernatant was filtrated through a 0.22-μm organic diameter filter (CLS8169, Sigma, Madrid, Spain) and transferred to Agilent (Barcelona, Spain) vials with glass inserts for further analysis.

5.5. Analysis conditions

The individual conditions for the detection and quantification of plasma metabolites are listed in Supplementary Table 2. For non-sulphur-containing metabolites, 2 μL of extracted sample was injected based on the method described (Method 1 [99]). Chromatographic separation was achieved on a reversed-phase column (Zorbax SB-Aq 2.1 × 50 mm, 1.8 μm particle size, Agilent Technologies, Barcelona, Spain) equipped with a pre-column (Zorba-SB-C8 Rapid Resolution Cartridge, 2.1 × 30 mm, 3.5 μm particle size, Agilent Technologies, Barcelona, Spain) with a column temperature of 60 °C. The flow rate was 0.6 mL/min during 19 min. Solvent A was composed of water containing 0.2% acetic acid (v/v) and solvent B was composed of methanol containing 0.2% acetic acid (v/v). The gradient started at 2% of solvent B and increased to 98% B in 13 min and held for 6 min. Post-time was established in 5 min. Electrospray ionization was performed in both positive and negative ion mode (depending on the target metabolite) using N_2 at a pressure of 50 psi for the nebulizer with a flow of 12 L/min and a temperature of 325 °C, respectively.

For sulphur-containing metabolites, 10 μL of extracted sample was injected based on the method described (Method 2 [100]). Chromatographic separation was achieved on a reversed-phase Supelcosil LC-CN column (Supelco of 4.6 × 250 mm, 5 μm particle size, Sigma, Madrid, Spain) with a column temperature of 30 °C. The flow rate was maintained at 0.5 mL/min during 10 min using a mobile phase of 10:90 acetonitrile/water with 0.1% formic acid (v/v). Electrospray ionization was performed in both positive and negative ion mode (depending on the target metabolite) using N_2 at a pressure of 50 psi for the nebulizer with a flow of 12 L/min and a temperature of 325 °C, respectively.

Data was collected using the MassHunter Data Analysis Software (Agilent Technologies, CA, USA). Samples were decoded and randomized before injection. Metabolite extraction quality controls (plasma samples with internal Phe-^{13}C) were injected every 10 samples. Peak determination and peak area integration were carried out with MassHunter Quantitative Analyses (Agilent Technologies, CA, USA).

5.6. Metabolite quantification

Metabolite quantification was performed by constructing standard curves for each metabolite. Serial dilutions of pure standards with internal Phe-^{13}C were prepared and processed following the methods described previously. Peak area normalized by internal Phe-^{13}C, and standard serial dilutions are used to construct the standard curves. Expected plasma concentration of each metabolite is based on the Human Metabolome Database (HMDB, http://www.hmdb.ca).

5.7. Equipment

The analysis was performed through liquid chromatography coupled to a hybrid mass spectrometer with electrospray ionization and a triple quadrupole mass analyser. The liquid chromatography system was an ultra-performance liquid chromatography model 1290 coupled to LC-ESI-QqQ-MS/MS model 6420 both from Agilent Technologies (Barcelona, Spain).

5.8. Statistics

Prior to statistical analyses, data was pre-treated (auto-scaled). Multivariate statistics were performed using Metaboanalyst software [101] (PCA, heatmaps) and caret [102] package from R [103] (PLS-DA). For PLS-DA, models were trained using a random subset of 60% of all the samples and were tested using the remaining 40%. Optimal number of components (1, 2 or 3) was selected according to the accuracy obtained from simple bootstrapping of the training samples. Among them, the model with higher accuracy was chosen. Model performance on test data was evaluated comparing the obtained accuracy with the no information rate accuracy (rate of the largest group) using a one-tailed binomial test. Univariate statistics were performed using GrapPhad Prism (v8.0.1). Pearson correlation, Pearson correlation matrix and network plots were performed using RStudio (v1.1.453). Correlation functions were included in the packages *Hmisc* [104]and *corrplot* [105], and plotted with *ggplot2* [106]. Network plot were constructed and plotted using the functions included in the package *igraph* [107].

Author contributions

J.V. and R.P designed the study. N.M.M., M.J., C.B., I.P., R.B., E.O., J. S., R.C., J.D.G.L. and J.P., performed experimental work. N.M.M, M.J., J. P., and R.P. analysed the data. R.P. supervised the design and data interpretation. The manuscript was written by N.M.M, M.J., C.B., J.V., and R.P. and edited by R.P. All authors discussed the results and commented on the manuscript.

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Declaration of competing interest

The 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.2020.11.026.

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