Modulation of Human Hydrogen Sulfide Metabolism by Micronutrients, Preliminary Data

Maurizio Dattilo1, Carolina Fontanarosa2,3, Michele Spinelli2,3, Vittorio Bini4 and Angela Amoresano2,3

1R&D Department, Parthenogen, Lugano, Switzerland. 2Department of Chemical Sciences, University of Napoli “Federico II,” Naples, Italy. 3Istituto Nazionale Biostrutture e Biosistemi, Rome, Italy. 4Department of Medicine and Surgery, Santa Maria della Misericordia Hospital, University of Perugia, Perugia, Italy.

ABSTRACT

BACKGROUND: Hydrogen sulfide (H2S) is a pivotal gasotransmitter networking with nitric oxide (NO) and carbon monoxide (CO) to regulate basic homeostatic functions. It is released by the alternative pathways of transulfuration by the enzymes Cystathionine Beta Synthase (CBS) and Cystathionine Gamma Lyase (CSE), and by Cysteine AminoTransferase (CAT)/3-Mercaptopyruvate Sulfur Transferase (3MPST). A non-enzymatic, intravascular release is also in place. We retrospectively investigated the possibility to modulate the endogenous H2S release and signaling in humans by a dietary manipulation with supplemented micronutrients (L-cystine, Taurine and pyridoxal 5-phosphate/P5P).

METHODS: Patients referring for antiaging purposes underwent a 10-day supplementation. Blood was collected at baseline and after treatment and the metabolome was investigated by mass spectrometry to monitor the changes in the metabolites reporting on H2S metabolism and related pathways.

RESULTS: Data were available from 6 middle aged subjects (2 women). Micronutrients increased 3-mercaptoppyruvate (P = .03), reporting on the activity of CAT that provides the substrate for H2S release within mitochondria by 3MPST, decreased lanthionine (P = .024), reporting the release of H2S from CBS, and had no significant effect of H2S release from CSE. This is compatible with a homeostatic balancing. We also recorded a strong increase of reporters of H2S-induced pathways including 5-MethylTHF (P = .001) and SAME (P = .022), reporting on methylation capacity, and of BH4 (P = .021) and BH2 (P = .028) reporting on nitric oxide metabolism. These activations may be explained by the concomitant induction of non-enzymatic release of H2S.

CONCLUSIONS: Although the current evidences are weak and will need to be confirmed, the effect of micronutrients was compatible with an increase of the H2S endogenous release and signaling within the control of homeostatic mechanisms, further endorsing the role of feeding in health and disease. These effects might result in a H2S boosting effect in case of defective activity of pathologic origin, which should be checked in duly designed clinical trials.

KEYWORDS: Cysteine, hydrogen sulfide, LC-MS/MS, l-cystine, micronutrients, pyridoxal 5-phosphate

RECEIVED: August 20, 2021. ACCEPTED: November 16, 2021.

TYPE: Original Research

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: MD reports personal fees from Parthenogen SAGL, outside the submitted work; in addition, MD is inventor of the pending patents “Dietary supplementation to achieve oxy-redox homeostasis and epigenetic stability” and “Combination of micronutrients to stimulate the endogenous production of hydrogen sulfide (H2S).” All other Authors have no competing interests to declare.

CORRESPONDING AUTHOR: Maurizio Dattilo, R&D Department, Parthenogen, Piazza Indipendenza 11, Lugano 6900, Switzerland. Email: maurizio.dattilo@parthenogen.ch

GRAPHICAL ABSTRACT: The administration of micronutrients appeared to increase H2S release from CAT/3MPST with a compensatory decrease from CBS. This associated to a strong activation of H2S dependent pathways suggesting the contribution from non-enzymatic production.
Introduction

Hydrogen sulfide (H$_2$S) is the third discovered gasotransmitter, besides nitric oxide (NO), and carbon monoxide (CO). It is known to cross-talk with the other 2 gasotransmitters and to modulate a variety of homeostatic functions by interacting with functional proteins by either persulfidation of their cysteine residues or interaction with the heme of metalloproteins. This results in the modulation of several homeostatic functions including endothelial function, coagulation, immunity, bioenergetics, and redox balance. 

A defective activation of H$_2$S release and of its signaling is strongly linked to a variety of pathologic conditions. These include endothelial dysfunction in both cardiovascular disease and erectile dysfunction, hypertension, insulin resistance and diabetes, neurodegeneration and autoimmune diseases. H$_2$S was also shown to exert anti-viral activity against a variety of pathogenic viruses and has been recently proposed as a defensive mechanism against SARS-CoV-2 and Covid 19. This has triggered enormous interest in the development of treatments boosting the H$_2$S signaling, which is currently concentrated on the use of either natural, chemical or pharmacologic donors whereas little information is available on the role of diet in modulating the H$_2$S signaling.

The substrate for H$_2$S endogenous production is cysteine resulting from protein catabolism or from endogenous synthesis, see Figure 1 for a global view of cysteine metabolism. Cysteine may generate H$_2$S by either enzymatic or non-enzymatic production. Three enzymes are known to release H$_2$S in physiologic metabolism. The best characterized release is operated by 2 enzymes belonging to the transsulfurations pathway, Cystathionine Beta Synthase (CBS), and Cystathionine Gamma Lyase (CSE), both requiring vitamin B6 in the form of pyridoxal 5-phosphosphate (P5P) as the necessary co-factor. The third H$_2$S releasing enzyme is 3-Mercaptopyruvate Sulfur Transferase (3MPST). This is not B6 dependent, however its activity is coupled to that of Cysteine AminoTransferase (CAT) producing 3-Mercaptopyruvate from cysteine and that is B6/P5P dependent. 3MPST generates H$_2$S mainly in mitochondria, which is followed by H$_2$S oxidation onto the respiratory chain to produce ATP. CBS/CSE and the transsulfuration pathway are responsible for the generation of cysteine from homocysteine, the catabolic product of methionine. CBS is a key enzyme mastering the partitioning of endogenous homocysteine between the re-methylation pathway on one side and its conversion to either cysteine/glutathione (GSH) or H$_2$S on the other side. When working according to the canonical pathway, CBS binds serine to homocysteine and forms cystathionine. This is cleaved by CSE to cysteine, which is then available for protein synthesis and for the de-novo synthesis of the universal cellular antioxidant GSH. CBS is redox-activated by the oxidation of its metal center but will be intensively cycling only after binding S-Adenosylmethionine (SAMe), when in excess from the methionine cycle, exerting an allosteric regulation on CBS. This ensures homocysteine transsulfuration, in alternative to its re-methylation, and generation of cystathionine feeding CSE to produce cysteine/GSH. However, both CBS and CSE exert ambiguous substrate recognition and may operate according to their alternative (reverse) pathways using cysteines to release H$_2$S. The stimuli to this functional shift are still subject of research, however both NO and CO can bind the CBS heme group inhibiting the canonical CBS activity, that is cysteine/GSH synthesis, while increasing the release of H$_2$S from CBS and, downstream, from CSE as the result of changed concentration of CSE substrates. It has been shown that H$_2$S release can be triggered by endoplasmic reticulum (ER) stress that induces the enzyme heme oxygenase (HO-1) to release CO as part of the Unfolded Protein Response (UPR). Binding of CO to CBS is followed by the activation of the alternative CBS pathway and release of H$_2$S. Finally, H$_2$S can also be generated non-enzymatically from cysteine by coordinated catalysis by Vitamin B6/P5P and iron to produce pyruvate, NH$_3$ and H$_2$S. The non-enzymatic release was proposed to occur into the bloodstream to regulate...
the background tone of the H₂S system. Being apparently less regulated, this pathway might exert stricter relation with the feeding behaviors.

In summary, cysteine is the main dietary substance supporting H₂S release, although also methionine consumption may contribute to cysteine availability via homocysteine. From a dietary point of view cysteine and its precursor methionine are considered equivalent, thus dietary assessments usually look at the sum of the 2 with no interest in the relative amounts. However, the metabolic signaling and dietary value of the 2 amino acids may be different. Methionine, which generates cysteine only when in excess on the demand for methylations, is more abundant in animal proteins with good contributions from cheese and wite meat. Opposite, cysteine, which is the direct substrate for H₂S release and is the least common amino acid in proteins, is better available from vegetable proteins including gluten. Thus, a diet enriched in vegetables is also enriched in cysteines, which may have relevant effects on H₂S endogenous metabolism and signaling. A recent, qualified position paper further endorsed the positive role of vegetable proteins in improving cardiovascular health based on epidemiologic data, but no background mechanism could be provided. Due to the enhanced content in cysteine of vegetable proteins, it is possible that an enhancement of H₂S signaling is involved in such protective effects. It is not known if an excess of dietary cysteines does actually result in such a metabolic adaptation, which prompted us at investigating this issue by means of a cysteine-based supplementation in human subjects.

Aiming to formulate such a supplement, we assumed that a straight increase of cysteine dietary intake might fail to increase free cellular cysteine concentration because any excess would be immediately removed by the enzyme cysteine dioxygenase (CDO) that catabolizes cysteine to hypotaurine, which is further oxidized to taurine, see Figure 1. Taurine exerts negative feedback on GSH synthesis,22 thus exerting a cysteine sparing effect and may help to increase free cysteines. Moreover, we also postulated that an excess of the end-product taurine could exert a negative biofeedback on CDO expression and/or activity and that an increased consumption of both cysteine and taurine would be synergic in increasing free cysteines. It was shown that taurine supplementation may increase circulating H₃S, which is likely explained by the above mechanisms. Finally, based on B₆/P₅P dependence of the H₃S releasing enzymes,24 we also postulated that supraphysiologic B₆ concentrations would further favor the use of cysteine in H₃S releasing reactions.

A dietary supplement had been formulated by including discrete amounts of L-cystine, as the physiologic dietary source of cysteines and taurine, as an enhancer of free cysteines. Supraphysiologic amounts of P₅P were added to direct cysteines to H₃S releasing reactions. The supplement had been administered to several patients within practices of precision medicine with antiaging purposes and its effects on the metabolome had been checked by mass spectrometry both before and after ten days of supplementation. Thus, we aimed at retrospectively analyzing the achieved metabolic changes to confirm the H₃S releasing activity of the supplement and to use the metabolic response as a probe for further understanding the role of dietary substances in H₃S metabolism in humans.

### Materials and Methods

#### Ethics

This was a retrospective study based on the outcomes from treatments with an approved dietary supplement within the standard clinical practice, therefore no ethical approval had been achieved. However, all patients released a written informed consent to the treatment, to the analyses and to the use of the data sourced as such, in anonymous form, for scientific purposes.

#### Patients and treatments

Several patients undergoing the micronutrient supplementation for antiaging purposes had been offered to verify the metabolic outcome of their supplementation by mass spectrometry and underwent the test between October 2019 and March 2020. Thereafter, due to the restrictions following the Covid 19 epidemic, the program was stopped. Therefore, few patients were available for our retrospective analysis. Moreover, our mass spectrometry analyses were performed on whole blood plasma without any derivatization or sample preparation: interferences and variable background effects were expected. Thus, aiming to minimize the possible discrepancies, we only included patients whose pre and post samples had been analyzed within the same run of mass spectrometry, which resulted in a small sample size.

All the included patients were free from any drug treatment and dietary supplementation since at least 1 month, had a good nutritional status and were following a balanced Western/Mediterranean dietary pattern. They were advised to avoid any change in their usual diet throughout the study period. In addition, each patient was asked to take note of the meals assumed on the day before the baseline sampling (number, time, and type of meals) and to follow, as far as possible, the same diet on the day before the second sampling. Each patient underwent a venous blood sampling at baseline. Plasma was prepared by centrifugation and stored at −20°C until analysis. Thereafter, each patient assumed 2 tablets of supplement by oral route 2 times daily during 10 days (a full box of 40 tablets). Each tablet contained 125 mg of L-cystine, 125 mg of taurine and 5 mg of P₅P (Redostim, Parthenogen, Switzerland). At the end of the 10-day treatment, the patients underwent a new blood sampling and, again, plasma was obtained and frozen. Both samples from each patient were analyzed in the same run of mass spectrometry.
**Liquid chromatography with tandem mass spectrometry (LC-MS/MS)**

**Chemicals and reagents.** All standards were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis and purchased from J.T. Baker (Phillipsburg, NJ).

**Preparation of standard solutions.** The stock solutions were prepared by adding 1.00 mL aliquots of each analyte to a 10-mL volumetric flask and bringing the standard to volume with methanol to yield a standard solution with 1000 µg/L of each analyte. The stock solutions were stored at −20°C until the analysis. Quantitative analysis was performed by construction of calibration curves for a set of standard molecules selected for the different class of analytes under investigation. Standard mixtures were prepared by series dilution as follows: 2.5, 5.0, 25, 50, 250, 500 µg/L.

**Sample preparation.** Serum from Vacutainer SST II Advance (BD Diagnostics 367 955, silica clotact/gel) was used. Samples (200 µL) were subjected to simple protein precipitation with (BD Diagnostics 367 955, silica clotact/gel) was used. Samples (200 µL) were subjected to simple protein precipitation with 250 µL of methanol and vortexed thoroughly. The mixture was stored at −20°C for about 30 minutes to complete protein precipitation and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was then directly transferred into HPLC auto sampler and 2 µl of supernatant was analyzed in a LC-MS/MS assay.

**LC–MS/MS instrumentation and conditions.** Two µl of supernatant were analyzed by using a 6420 triple quadrupole system with a HPLC 1100 series binary pump from Agilent Technologies. The mobile phase was generated by mixing eluent A (0.1 % Formic Acid in water) and eluent B (0.01 % Formic Acid in methanol) and the flow rate was 0.200 mL/minute. Chromatographic gradient was from 5% to 70% in 2 minute and then raised to 95% eluent B in 1 minute. Tandem mass spectrometry was performed using a turbo ion spray source operated in positive and negative ion mode, and the multiple reaction monitoring (MRM) mode was used for the selected analytes. A standard solution of 500 pg/µL of each metabolite was used for optimization of the MRM transition. Metabolites were automatically (flow injection) tuned for ionization polarity, product ion, and collision energy (CE) using metabolite standard solutions via Agilent MassHunter Optimizer software. Supplemental Table 1 provides a list of precursor ion, product ions, collision energy, and polarity.

**Data processing.** Extracted mass chromatogram peaks of metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00). Peak areas of corresponding metabolites are then used, as quantitative measurements, for assay performance assessments such as assay variation and linearity.

**Quantification of analytes.** The first step for the setting of mass spectral analysis consisted in the MRM detection of the analytes individually infused to establish the optimal instrument settings for each compound. Experimental automatic tuning by using MassHunter Optimizer was used to define ionization polarity, to select the best product ion (Q3 ion) and to optimize both the collision energy (CE) and the declustering potential (DP). The MRM transitions and all the instrumental parameters determined are summarized in Supplemental Table 1. Standard calibration curves for the selected set of molecules, were constructed by plotting peak areas against concentration (pg/µL), and linear functions were applied to the calibration curves. Data were integrated by Mass Hunter quantitative software showing a linear trend in the calibration range for all molecules. The coefficients of determination (R2) were greater than 0.99 for all analytes. Analytical quantification parameters are summarized in Supplemental Table 2. Once set up, the MRM mass spectral method was applied to the analyses of specific sets of serum samples.

**Statistical analyses**

The data are presented as means ± SD in the text whereas tables and figures do not list SDs to facilitate their reading. However, all the SDs are duly reported in Supplemental Table 3.

Due to the small sample size, we could not assess the normal distribution of the data and used the Wilcoxon signed rank test to analyze paired data. However, although not visible in the current dataset, the concerned variables are known to undergo a normal distribution and were also analyzed by the paired sample T-test. The outcomes from both tests have been reported in the text: “W” marks the Wilcoxon signed rank test P value whereas “T” indicates the P values according to T-test. In figures we only reported the outcome of the T-test. Data analysis was performed using IBM-SPSS® version 26.0. In spite of the multiplicity of the tests performed (50 variables), being this an explorative study, for further investigation, no correction of experiment-wise error rates in multiple comparisons has been applied, and a two-sided P value <.05 was considered significant in all analyses.

**Results**

Data from both pre and post treatment obtained within the same run of mass spectrometry were available from 6 patients, (2 females), aged between 55 and 62 years. A total of 46 different metabolites was detected and quantified, although the data for 3 metabolites (5-MethylTHF, methylcobalamin, and sarcosine) were missing in 4 patients whose test had not yet included the due standards. The mean values of all the tested variables before and after treatment are depicted in Table 1 whereas the outcomes in the different metabolic domains are described in dedicated paragraphs here below. The full set of individual data is available as Supplemental Table 3.
Table 1. Metabolic changes following micronutrient administration.

| ANALYTES                          | PRE TREATMENT | POST TREATMENT | % CHANGE | P VALUE      | WILCOXON | T-TEST |
|-----------------------------------|---------------|----------------|----------|--------------|----------|--------|
|                                   | MEAN          | SD             | MEAN     | SD           |          |        |
|                                   | MEAN          | SD             | % CHANGE |              |          |        |
| 10-FormylTHF                      | 0.52          | 0.19           | .51      | 0.17         | -0.0     | .9165  |
|                                   |               |                |          |              |          | .9310  |
| 3-Mercaptopruvate                 | 0.27          | 0.08           | .39      | 0.14         | 45.7     | .0464  |
|                                   |               |                |          |              |          | .0300  |
| 5,10-MethenylTHF                  | 0.65          | 0.12           | .83      | 0.14         | 28.2     | .0458  |
|                                   |               |                |          |              |          | .0470  |
| 5,10-MethyleneTHF                 | 0.86          | 0.15           | .74      | 0.20         | 11.9     | .3454  |
|                                   |               |                |          |              |          | .4940  |
| 5-MethylTHF                       | 17.38         | 8.89           | 2.08     | 8.89         | 15.5     | .1573  |
|                                   |               |                |          |              |          | .0010  |
| Adenosine                         | 28.19         | 4.07           | 34.98    | 6.36         | 24.1     | .0277  |
|                                   |               |                |          |              |          | .8100  |
| Arginine                          | 11.570        | 2789           | 12.076   | 5930         | 4.4      | .9165  |
|                                   |               |                |          |              |          | .8100  |
| Asymmetric dimethylarginine       | 28.994        | 7.807          | 29.237   | 8.029        | 0.8      | .9139  |
|                                   |               |                |          |              |          | .4240  |
| Betaine                           | 202.86        | 52.65          | 302.86   | 289.32       | 49.3     | .4618  |
|                                   |               |                |          |              |          | .3610  |
| BH2                               | 2.66          | 1.41           | 5.55     | 2.91         | 108.9    | .0277  |
|                                   |               |                |          |              |          | .0277  |
| BH4                               | 3.39          | 1.86           | 7.08     | 3.42         | 108.8    | .0277  |
|                                   |               |                |          |              |          | .0210  |
| Ratio BH4: BH2                    | 1.35          | 0.49           | 1.52     | 0.89         | 13.0     | .9165  |
|                                   |               |                |          |              |          | .7140  |
| Biliverdin                        | 3.53          | 0.70           | 3.92     | 1.89         | 11.2     | .6002  |
|                                   |               |                |          |              |          | .4850  |
| Choline                           | 733.84        | 457.21         | 292.50   | 254.11       | -60.1    | .1159  |
|                                   |               |                |          |              |          | .0750  |
| Citrulline                        | 5.22          | 1.82           | 5.41     | 1.42         | 3.6      | .6002  |
|                                   |               |                |          |              |          | .6002  |
| Cystathionine                     | 5.78          | 3.05           | 4.30     | 4.35         | -25.7    | .2476  |
|                                   |               |                |          |              |          | .2910  |
| Cysteine                          | 15.500        | 6.575          | 34.616   | 26.321       | 123.3    | .0273  |
|                                   |               |                |          |              |          | .1590  |
| DiHydrofolate (DHF)               | 0.23          | 0.07           | .40      | 0.28         | 70.6     | .1730  |
|                                   |               |                |          |              |          | .1730  |
| Dimethyl-glycine                  | 317.96        | 160.95         | 452.40   | 302.27       | 42.3     | .2489  |
|                                   |               |                |          |              |          | .1500  |
| Folate                            | 12.92         | 2.97           | 13.23    | 4.40         | 2.5      | .6002  |
|                                   |               |                |          |              |          | .6420  |
| Formate                           | 11.87         | 2.48           | 14.15    | 5.09         | 19.2     | .1730  |
|                                   |               |                |          |              |          | .1540  |
| γ-glutamylcysteine                | 67.12         | 69.74          | 109.34   | 64.33        | 62.9     | .0273  |
|                                   |               |                |          |              |          | .0700  |
| Glutamate                         | 3.625         | 1.740          | 4.041    | 1.668        | 11.5     | .3454  |
|                                   |               |                |          |              |          | .5380  |
| Glycine                           | 12.204        | 3.530          | 6.216    | 7.482        | -49.1    | .1159  |
|                                   |               |                |          |              |          | .0310  |
| GSH (reduced glutathione)         | 0.73          | 0.07           | .62      | 0.58         | -14.7    | .6002  |
|                                   |               |                |          |              |          | .6830  |
| GS-SG (Oxidized glutathione)      | 0.07          | 0.01           | .07      | 0.05         | 0.8      | .7532  |
|                                   |               |                |          |              |          | .9810  |
| Ratio GSH:GSSG                    | 10.11         | 0.59           | 7.75     | 4.89         | -23.4    | .2489  |
|                                   |               |                |          |              |          | .2620  |
| Guanidinoacetate                  | 121.90        | 25.21          | 124.45   | 25.69        | 2.1      | .3454  |
|                                   |               |                |          |              |          | .5800  |
| Homocysteine                      | 1.771         | 656            | 1.534    | 482          | -13.1    | .1730  |
|                                   |               |                |          |              |          | .1740  |
| Homolanthionine                   | 0.70          | 0.12           | .78      | 0.19         | 11.1     | .3441  |
|                                   |               |                |          |              |          | .2400  |
| Homoserine                        | 3.464         | 847            | 3.114    | 700          | -10.1    | .4631  |
|                                   |               |                |          |              |          | .3360  |
| Hypotaurine                       | 3.88          | 1.75           | 3.03     | 0.63         | -22.0    | .1148  |
|                                   |               |                |          |              |          | .1510  |
| Lanthionine                       | 6.06          | 2.06           | 3.93     | 1.00         | -35.2    | .0464  |
|                                   |               |                |          |              |          | .0240  |
| Methionine                        | 819.93        | 413.26         | 1.264    | 845          | 54.2     | .1148  |
|                                   |               |                |          |              |          | .2100  |
| Methylcobalamin                   | 0.28          | 0.03           | .64      | 0.03         | 130.7    | .1573  |
|                                   |               |                |          |              |          | .0001  |

(Continued)
Administered substances

The trend for increased post-treatment concentration of the micronutrients contained in the test product (Redostim, Parthenogen, Switzerland) support the idea that they were actually bioavailable. P5P, which was administered at a total dose of 20 mg per day, non-significantly increased by 20% whereas taurine (total daily dose 500 mg) did not change (−1.6%). Cysteine concentration (total daily dose 500 mg of L-cystine), which is very stable without interventions, increased by 126% from 15 500 ± 6575 to 34 616 ± 26 321 μg/L. This increase was significant only according to the Wilcoxon signed rank test (P = .027).

Transsulfurations pathway: Effects on H2S and GSH metabolism

The effect of the micronutrients on H2S metabolism was evaluated by checking the changes in the concentration of the end-products of H2S releasing reactions. In particular, we assumed lanthionine to be the main reporter of H2S release form CBS reactions and homolanthionine to report on the release from CSE reactions.1 The activity of the CAT/3MPST pathway was evaluated looking at changes in 3-mercaptopyruvate, reporting about 3MPST reaction, and sulfates (SO42−) generated by final H2S oxidation onto the respiratory chain.24,29 The main changes of these metabolites are depicted in Figure 2.

The concentration of lanthionine, marking H2S release from CBS, significantly decreased (−35.2%) from 6.06 ± 2.06 to 3.95 ± 1.00 μg/L (W: P = .046; T: P = .024). We also recorded a non-significant 11% increase of homolanthionine, reporting on H2S release from CSE activity, from 0.70 ± 0.12 to 0.76 ± 0.19 μg/L. The concentration of 3-mercaptopyruvate, reporting on the activity of CAT, significantly increased (+45.6%) from 0.27 ± 0.08 to 0.39 ± 0.14 μg/L (W: P = .046; T: P = .03). The increase of pyruvate, reporting on intra-mitochondrial 3-mercaptopyruvate hydrolysis by 3MPST, was small (3.6%) but showed a trend for significance (from 3582 ± 5934 to 3713 ± 623 μg/L; W: P = .067; T: P = .052) whereas sulfates reporting on H2S oxidation in the generation of ATP non-significantly increased by 5.5%.

GSH metabolism was also changed by the supplement. The concentration of the GSH precursor γ-glutamylcysteine significantly increased (+62.9%) from 67.12 ± 69.7 to 109.34 ± 64.3 (W: P = .027; T: P = .07). Nevertheless, GSH non-significantly decreased (−14.7%) from 0.72 ± 0.70 to 0.62 ± 0.58 and the GSH to oxidized glutathione (GSSG) ratio also decreased non significantly from 10.1 to 7.75.

In summary, following micronutrient supplementation, the changes in the markers were compatible with a decreased

Table 1. (Continued)
release of H₂S from CBS, the markers of CSE activity showed little, non-significant positive changes whereas the concentration of 3-mercaptopyruvate from CAT increased, which is compatible with increased H₂S release from 3MPST.

**Effects on one carbon metabolism/methylations**

Micronutrients strongly activated the one carbon metabolism, see Figure 3.

Metabolites reporting on the folate cycle, feeding homocysteine re-methylation from folates, were increased, which reached significance for 5,10-methylenetTHF (+28.2%) from 0.65 ± 0.12 to 0.83 ± 0.14 µg/L (W: P = .046; T: P = .047) and for 5-MethylTHF (+15.5%) from 17.39 ± 8.9 to 20.08 ± 8.9 µg/L (W: P = .157; T: P = .047). The alternative homocysteine remethylation pathway using betaine generated within mitochondria was also upgraded. However, the increase of betaine (+49.3%) and of its de-methylation product dimethylglycine (+42.3%) was not significant. Nevertheless, the reduction of choline (−60.1%) from 733.84 ± 457.2 to 292.50 ± 254.11 µg/L showed a trend for significance (W: P = .116; T: P = .075) and may reflect increased intramitochondrial generation of betaine from choline. Accordingly, we recorded a significant increase of the reporters of the methionine cycle and of methylation capacity. SAMe was significantly increased (+25%) from 2.51 ± 0.75 to 3.14 ± 0.93 µg/L (W: P = .027; T: P = .022) as well as the ratio of SAMe to S-Adenosylhomocysteine (SAHo) (+23.6%) from 0.82 ± 0.11 to 1.02 ± 0.17 (W: P = .046; T: P = .032). This indicates increased cycling because coupled with a significant increase (+24%) of the SAHo de-adenosylation product, adenosine. Moreover, methylcobalamin (CH₃-B₁₂), which is the specific activated form of vitamin B₁₂ responsible for the final passage of methyl groups from 5-MethylTHF to homocysteine, was significantly increased (+130.7%) from 0.27 ± 0.03 to 0.64 ± 0.03 µg/L (W: P = .157; T: P = .0001). Worth to note from a safety point of view, the upgraded betaine metabolism did not cause an increase of trimethylamine oxides (TMAO, −4.1%), likely due to the compensatory decrease of choline.

**Effects on NO and CO metabolism**

The changes of metabolites within the NO and CO pathways are depicted in Figure 4.

The administration of micronutrients resulted in a massive increase of both reduced (BH₄) and oxidized (BH₂) biopterins. BH₄ significantly increased (+108.8%) from 3.39 ± 1.86 to 7.08 ± 3.42 µg/L (W: P = .028; T: P = .021) as well as BH₂ (+108.9%) from 2.66 ± 1.41 to 5.55 ± 2.91 µg/L (W: P = .028; T: P = .028). A positive BH₄ to BH₂ ratio is necessary for the synthesis of NO and it increased by 13.4% from 1.35 ± 0.49 to 1.52 ± 0.89, although this difference was not significant. The concentration of arginine (+4%), substrate for NO release, and of citrulline (+3.6%), end product after NO release did not change, that is they did not accumulate confirming that NO synthesis was well cycling. This was also confirmed by no change (+0.8%) of asymmetric dimethylarginine, the toxic by-product from arginine accumulation. There was also a significant increase of ornithine (+4.4%) from 4308.50 ± 734.2 to 4497.15 ± 717.7 µg/L (W: P = .024; T: P = .0001) which might indicate a boost to the putrescine/polyamine pathway. In summary, the recorded changes suggest a strong activation of NO metabolism (increased BH₄ and BH₂) with a prevalence of a coupled nitric oxide synthase reaction (increased BH₄:BH₂ ratio) and no signs of accumulation of substrates or end-products.

We had limited capacity to monitor the effects of the micronutrients on CO metabolism. Iron and ferritin, produced together with CO during the heme catabolism by HO-1, are...
involved in many other homeostatic regulations and were not targeted. Biliverdin, which can be assumed as a specific marker of the reaction, non-significantly increased (+11.2%) from 3.53 ± 0.70 to 3.92 ± 1.89. This change is compatible with a boosting effect of the micronutrients on this pathway.

Discussion
This is, to our knowledge, the first study attempting at measuring the effects of dietary manipulations of the H2S releasing pathways in humans by looking at the metabolome by mass spectrometry. LC–MS/MS confirmed to be a suitable method for investigating the human metabolome dosing all the target analytes in 1 shot without any sample preparation or derivatization. However, due to possible interferences between substances and unknown background effects we had to restrict our sample to those patients tested before and after micronutrient exposure within the same MS run, which limits the clinical suitability of this approach. Moreover, for the same reasons, the calculated amounts of the single metabolites may not precisely report their actual concentration and we could...
Our findings support the hypothesis that supraphysiologic exposure to P5P further pushes in the same direction.

The micronutrients appeared to upgrade the CAT/3MPST pathway, which is known to work mainly within mitochondria where the released H₂S can be oxidized onto the respiratory chain to contribute to ATP production. They had little (positive) effect on CSE reactions and significantly depressed the CBS alternative pathway. This is likely reflecting the different and increasing B6 sensitivity of, respectively, CBS, CSE, and CAT (3MPST pathway). However, other mechanisms might contribute.

The CBS alternative pathway is likely the one undergoing the strictest regulation so to be activated only at time of actual need. The best described trigger for this shift is the occurrence of ER stress activating HO-1 to release CO. This binds the heme center of CBS inhibiting its canonical activity, thus allowing the alternative pathway to work. Our patients were not known to suffer ER stress; Thus, they did not undergo signals for activation of CBS alternative pathways. We speculate that the increased release of H₂S from other pathways gave negative feedback to the production from CBS, that is, the micronutrients exerted their action under the control of metabolic homeostasis. It remains to be assessed if the same micronutrients would upgrade also the CBS pathway if it was needed, that is, if ER stress was in place.

CSE has been shown to release H₂S mainly by using 2 homocysteines to generate homolanthionine, that is, to be involved in the reaction to hyper-homocysteinemia. Our patients did not have high homocysteine and this reaction was unlikely to increase under micronutrient supplementation and to contribute to the non-significant 11% increase of homolanthionine that we recorded. However, CSE can also produce homolanthionine when releasing H₂S from cystathionine, the product of CBS canonical reaction leading to cysteine/GSH synthesis. Cystathionine non-significantly decreased after treatment by 26%, which is unlikely to result from consumption for cysteine synthesis, due to the increased cysteine level after supplementation. Therefore, it is likely that cystathionine was consumed to produce H₂S, which may account for the increased homolanthionine.

The upgrade to the CAT pathway was unquestionable. The reaction product of cysteine with α-ketoglutarate, 3-mercaptopropionivat, was strongly and significantly increased by 45%, possibly due to the high sensitivity of CAT to increased availability of P5P and free cysteines. Downstream, the reaction product of 3MPST, pyruvate, and of oxidation of H₂S to produce ATP, sulfate, were also increased, although only pyruvate increase was of borderline significance and with modest changes from baseline. However, pyruvate is consumed in a variety of reactions and accumulation may be difficult to occur. Sulfates have high degree of protein binding and are rapidly eliminated in the urine so that little changes of their blood level are expected in physiologic conditions. They may accumulate in urine, but this had not been checked in our patients.

H₂S produced by 3MPST can also be stored in the form of protein bound sulfane sulfur. This is hexavalent and uncharged sulfur that covalently and reversibly binds other sulfur atoms, typically free or protein bound cysteines. Sulfane sulfur can be accumulated/stored to be mobilized and provide protein persulfidation signaling or ATP synthesis in situations of
increased reducing conditions, especially in the brain. Thus, sulfane sulfur storages may be a tool to locally increase \( \text{H}_2\text{S} \) from steady state to effective concentration, that is it could be the main determinant for the onset of \( \text{H}_2\text{S} \) signaling.\(^{14}\) Storage as sulfane sulfur of the \( \text{H}_2\text{S} \) produced by 3MPST in our patients may explain why the significant increase of 3-mercaptoppyruvate was not paralleled by a similar increase of \( \text{SO}_\text{4}^- \), that is most of the \( \text{H}_2\text{S} \) released was not used for ATP synthesis (no need) but stored as sulfane sulfur. Thus, if taken for longer time, the supplementation or its equivalent from diet, might be effective in replenishing the sulfane sulfur storages and in increasing the ability to activate \( \text{H}_2\text{S} \) signaling at time of need.

In summary, our results suggest that the excess of cysteine provided by the supplement had been disposed of by using it in mitochondria for bioenergetic purposes and/or to replenish sulfane sulfur storages. It is important to note that CAT activity depends on the occurrence of hydrolysis of L-cysteine (Cys-S-S-Cys) to release free cysteines and this reaction is itself GSH dependent. Therefore, the activity of CAT is homeostatically limited by GSH availability.\(^{35}\)

In this respect, the changes occurred to glutathione metabolism following micronutrients are intriguing. In spite of increased cysteines, both GSH (-15\%) and total glutathione (-13\%) non-significantly decreased with a larger, non-significant decrease (-23\%) of the ratio of GSH to GSSG, which means a shift to a less reducing balance and lower ability to hydrolyze L-cysteine to free cysteines. This may have been a limit for further growth of 3-mercaptoppyruvate. Accordingly, the activity of the micronutrients is somehow self-limited, or homeostasis controlled, and unlikely to produce a toxic excess. On the other side, the non-significant decrease of GSH is to be seen in parallel with the increased disposition of SH groups, that is, reducing power, in bioenergetic reactions. These changes synergize in moving the redox balance toward less reducing conditions.

Based on rodent in vivo data, the reaction of glutamate cysteine ligase (GCL) is currently considered the rate limiting and regulated step in GSH synthesis.\(^{30}\) However, in our patients the partial reduction of GSH associated to a significant accumulation of the GCL product \( \gamma \)-glutamylcysteine (+63\%). This indicates that in our patients the regulated step for GSH synthesis was GSH synthetase (GS). Although this regulation might have been activated by our dietary manipulation, the enzymes of the 1 carbon metabolism are described to behave in rodents in a different manner\(^{17}\) and differences in regulation of GSH synthesis between humans and rodents are possible.

We also looked at the effect of the micronutrients on the \( \text{H}_2\text{S} \)-related pathways so to individuate any negative counter-changes and to argue on the occurrence of \( \text{H}_2\text{S} \)-related effects. The 1 carbon metabolism, that is the source of activated carbon units for transmethylation reactions, was strongly upgraded as doubtless proven by an increased ratio of \( \text{SAMe} \) to \( \text{SAHo} \) and by the significant increase of the de-adenylation product of \( \text{SAHo} \), adenosine. Upstream, the concentration of methionine also grew and the pathways generating the methyl donors for homocysteine re-methylation (folate cycle and betaine cycle) were significantly activated. Among these changes, we recorded a surprising massive increase (+130\%) of methylcobalamin. This finding is based on only 2 patients tested and the change was significant only based on the \( Z^-\text{test} \) \((P<.0001)\). However, it seems to report a massive activation of the \( \text{H}_2\text{S} \) release. Several research evidences point to a role of the sulfur atom in the B12-dependent transfer of methyl groups and a specific model for the interaction between \( \text{H}_2\text{S} \) and cobalamin, leading to the generation of methylcobalamin, had been proposed.\(^{38}\)

Our findings seem to endorse that model. This mechanism could be an explanation for the effect of micronutrients on the whole methylation pathway. In the nitric oxide pathway, we recorded a massive, significant increase (+109\%) of both the reduced (tetrahydrobiopterin—BH4) and the oxidized (dihydrobiopterin—BH2) forms of bioppterins. Nitric oxide synthases (NOSs) use bioppterins in the conversion of arginine to citrulline. During this passage only NOS coupled with BH4 is able to produce NO whereas NOS coupling with BH2 results in the oxidation of \( \text{O}_2 \) to generate superoxide anion (\( \text{O}_2^- \)).\(^{39}\) Locally released \( \text{O}_2^- \) can contribute to BH4 oxidation to BH2 thus creating a vicious loop leading to NO deficiency with (decreased cycling) or without (normal cycling) arginine accumulation. In our patients, arginine did not accumulate, neither did citrulline, which can be interpreted as intensive/normal cycling. Although non-significant, the increase (+13\%) of the BH4 to BH2 ratio vouches for NO as the product of such sustained cycling. Interestingly, downstream to citrulline, we recorded a significant increase of ornithine that may indicate an upgraded polyamine metabolism, which was however not checked in our patients. The increased concentration of BH4 and BH2 is likely due to increased synthesis. However, experiments with cell cultures demonstrated that the application of \( \text{H}_2\text{S} \) donor rapidly and strongly activated the endothelial NOS, which appeared as a direct effect.\(^{40}\) The same may have happened in our patients, that is, there may have been a direct effect of \( \text{H}_2\text{S} \) in the reduction of BH2 to BH4.

Previous studies demonstrated the role of \( \text{H}_2\text{S} \) as an endogenous, physiologic inhibitor of phosphodiesterase 5 (PDE5)\(^{41}\) and this activity is currently seen as a main explanation of the protective role of \( \text{H}_2\text{S} \) in various forms of endothelial dysfunction.\(^2\) This was not evaluated in our patients, however the increased NO production here induced by micronutrients, if actually coupled with an increased inhibition of PDE5 from increased \( \text{H}_2\text{S} \) release, might reach clinical relevance in the treatment of endothelial dysfunction.

The interaction between \( \text{H}_2\text{S} \) and HO-1/CO metabolism is bi-directional. CO released from HO-1 can induce \( \text{H}_2\text{S} \) release
by activating the CBS alternative pathway. In turn, \( \text{H}_2\text{S} \) was shown to induce the expression of \( \text{HO}-1 \) mRNA and protein both in rodent and in human cells. Experiments based on the application of \( \text{H}_2\text{S} \)-donors in murine cells showed that \( \text{H}_2\text{S} \) is also able to modify the activity of \( \text{HO}-1 \), rendering it less oxygen dependent, thus improving the \( \text{HO}-1 \) response to extreme hypoxia. However, our patients did not have known reasons to activate \( \text{HO}-1 \) and a strong activation of this pathway was therefore not expected. Indeed, we recorded a relatively small and non-significant increase of biliverdin (+11%) that is compatible with some \( \text{HO}-1/\text{CO} \) activation but, in any case, to a low extent. However, it remains possible that the increased background availability of \( \text{H}_2\text{S} \) induced by micronutrients would facilitate and/or accelerate the activation of \( \text{HO}-1 \) at time of need, for example, during transient tissues hypoxia or at time of ER stress. This activity, if confirmed, might be of relevance in the activation of innate immunity, in the reaction to SARS-Cov2 infection and in the treatment of Covid-19.

It has been recently shown in murine in-vivo and ex-vivo models that \( \text{H}_2\text{S} \) can be released, together with pyruvate and \( \text{NH}_3 \), in blood at physiologic pH and oxygen tension, by non-enzymatic reactions involving cysteine, vitamin B6 in the form of \( \text{P}5\text{P} \) and ferric iron. The reaction was shown to be both \( \text{P}5\text{P} \) and \( \text{Fe}^{3+} \) dose dependent, specifically required vitamin B6 in its \( \text{P}5\text{P} \) form and free cysteines whereas L-cystine did not work and acetylcysteine inhibited the reaction. Due to little variability in vivo of the \( \text{P}5\text{P} \) level, the Authors claimed for a main physiopathological role of this pathway in hemolytic conditions resulting in the release of free or heme-bound iron. Their study did not check the effect on the reaction of escalating concentration of cysteine, however it used cysteine 10 millimolar, which is high. Our patients were exposed to both high cysteines and supraphysiological \( \text{P}5\text{P} \), therefore intravascular, non-enzymatic release of \( \text{H}_2\text{S} \) was expected. However, we cannot prove it because the only possible metabolic marker, pyruvate, that had in our patients a small change (+3%) but with a trend for significance, is unlikely to be a precise reporter because involved in a variety of compensating reactions. However, the massive activation of methylation and NO metabolism recorded in our patients suggests a release of \( \text{H}_2\text{S} \) that seems stronger than that assumed on the basis of the \( 3\text{MPST} \) increase alone. Therefore, some non-enzymatic intravascular release from cysteine was likely to be taking place. Moreover, experiments with mouse aortic rings in vitro clearly showed that 3-mercaptoppyruvate may itself serve as a substrate for \( \text{H}_2\text{S} \) non-enzymatic release. The study did not detail all the molecules involved in the reaction, however 3-mercaptoppyruvate has the potential to substitute cysteine in the co-ordinated catalysis by \( \text{P}5\text{P} \) and iron. Thus, 3-mercaptoppyruvate may be a system to export to the bloodstream amounts of \( \text{H}_2\text{S} \) produced into cells, and this may have happened in our patients. In any case, pending a better understanding of the mechanism, the present data suggest that micronutrients may have supported increased intravascular release of \( \text{H}_2\text{S} \) and this may have clinical relevance in the treatment of endothelial dysfunction.

The next question is whether the metabolic changes here described may actually occur following a standard dietary change, that is without a supplement, and if they could be involved in the protective effects from a diet imbalanced toward vegetable proteins, which are enriched in cysteines. Our subjects assumed a supraphysiologic dose of vitamin B6, that is 20mg compared to the standard daily human requirement of 1.4mg, and this amount is unlikely to be achieved by consuming standard meals. Moreover, B6 is well provided by meat and fish with lower availability from vegetables, the best sources of cysteines. Taurine also likely contributed to the effect and, similarly to B6, it is mainly provided by fish and meat with negligible content in vegetables. On this base, a significant enhancement of enzymatic \( \text{H}_2\text{S} \) metabolism and signaling from a vegetarian diet is unlikely. However, in the present study the main source of \( \text{H}_2\text{S} \) release appeared to result from non-enzymatic intravascular release further endorsing the sensitivity of this pathway to the dietary inputs. Thus, in subjects with an adequate B6 and iron status, an increase of dietary cysteines might be still able to induce a subtle enhancement of non-enzymatic intravascular release of \( \text{H}_2\text{S} \) and to contribute to the enrichment of the sulfane sulfur storages. Thus, in the long run, a positive effect from a cysteine-enriched diet is possible and the dietary patterns may have an effect on \( \text{H}_2\text{S} \) signaling. However, our data point to the potential benefits from a well-balanced diet, providing good amounts of B6, taurine and iron (meat and fish) together with cysteines (vegetables), and not to a straight vegetarian diet.

**Conclusions**

We have shown that the metabolic changes resulting from a micronutrient support containing L-cystine, taurine and supraphysiologic amounts of activated vitamin B6 (\( \text{P}5\text{P} \)) is likely to be effective in inducing an increased endogenous release of \( \text{H}_2\text{S} \) and to do so within the control of homeostatic mechanisms. Our study carries several methodological weaknesses, including the small sample and the retrospective nature, and prospective data are needed to confirm these findings and the suitability of this approach for the correction of clinical conditions with defective \( \text{H}_2\text{S} \) release. We recorded strong secondary upgrading effects on the methylation pathways and on NO metabolism that are compatible with \( \text{H}_2\text{S} \) induction, thus confirming in-vivo, in humans and in physiologic conditions, previous experimental findings and providing evidences for new possible regulating mechanisms. The magnitude of upregulation of methylation and NO metabolism went beyond the one expected based on the recorded increase of the markers of \( \text{H}_2\text{S} \) enzymatic production and part of the effect was likely achieved by non-enzymatic intravascular release, which may have clinical applications in endothelial dysfunction. Similar, positive metabolic adaptations could be sustained by a balanced diet.
comprising both animal/fish and vegetable food, which needs to be explored in purposely designed studies.

The investigation of the metabolome from unprocessed blood plasma by LC-MS/MS confirmed to be a suitable methodology to understand the actual outcomes from dietary interventions and to provide meaningful insights into the backing mechanisms. Accordingly, it might be considered as a standard practice in the validation of nutritional interventions for the clinical use.

**Author Contributions**
MD: Study design, interpretation of the data and manuscript editing; CF and MS: LC-MS/MS set-up, analyses and manuscript editing; VB: study design, statistical analysis and manuscript editing; AA: study design, LC-MS/MS set-up and manuscript editing.

**Availability of Data and Materials**
Supplemental Table 1 provides full information on LC-MS/MS conditions. Supplemental Table 2 depicts the analytical quantification parameters. Supplemental Table 3 provides all individual data for all the tested metabolites and their mean values ± standard deviation.

**Ethics Approval and Consent to Participate**
This is a retrospective study based on the outcomes from treatments within the standard clinical practice with an approved dietary supplement, therefore no Ethical approval had been achieved. All patients released a written informed consent to the treatment, to the analyses and to the use of the data sourced as such, in anonymous form, for scientific purposes.

**Consent for Publication**
All the authors read and approved the final manuscript.

**ORCID iD**
Maurizio Dattilo [https://orcid.org/0000-0002-0676-7929](https://orcid.org/0000-0002-0676-7929)

**Supplemental Material**
Supplemental material for this article is available online.

**REFERENCES**

1. Giuffré A, Vicente JB. Hydrogen sulfide biochemistry and interplay with other gaseous mediators in mammalian physiology. *Oxid Med Cell Longev*. 2018;2018:6299531.

2. Cin V, Martelli A, Goricza E, Brogi S, Testai L, Calderone V. Role of hydrogen sulfide in endothelial dysfunction: pathophysiology and therapeutic approaches. *J Adv Res*. 2021;27:99-113.

3. d’Emmanuele di Villa Bianca R, Sorrentino R, Mirone V, Cirino G. Hydrogen sulfide: a gaseous mediator in mammalian physiology. *Oxid Med Cell Longev*. 2014;111:E3845-E3852.

4. Singh S, Padavani D, Leslie RA, Chiku T, Banerjee R. Relative contributions of Cystathionine beta-synthase and gamma-cystathionase to H₂S biogenesis via alternative trans-sulfuration reactions. *J Biol Chem*. 2009;284:22457-22466.

5. Banerjee R. Catalytic promiscuity and heme-dependent redox regulation of H₂S synthesis. *Curr Opin Chem Biol*. 2017;37:115-121.

6. Shefa U, Kim M-S, Jeong NY, Jung J. Antioxidant and cell-signaling functions of hydrogen sulfide in the central nervous system. *Oxid Med Cell Longev*. 2018;2018:1873962.

7. Dilek N, Papapetrosou A, Tolvanen-Kinsky T, Szabo C. Hydrogen sulfide: an endogenous regulator of the immune system. *PharmacoRes*. 2020;141:105119.

8. Kazhanov N, Ivanucci T, Wu H, et al. Thiol activated hydrogen sulfide donors antiviral and anti-inflammatory activity in respiratory syncytial virus infection. *Virus*. 2018;10:249.

9. Dattilo M. The role of host defences in covid 19 and treatments thereof. *Med Mol*. 2020;26:980.

10. Yang G. H₂S as a potential defense against COVID-19. *Am J Physiol Cell Physiol*. 2020;319:C244-C249.

11. Bourgonje AR, Offerings AK, van Eijk LE, et al. N-Acetylcysteine and hydrogen sulfide in coronavirus disease 2019. *Antioxid Redox Signal*. 2021;31:1207-1225.

12. Powell CR, Dillon KM, Matson JB. A review of hydrogen sulfide (H₂S) donors: chemistry and potential therapeutic applications. *Biochem Pharmacol*. 2018;149:110-123.

13. Szabo C, Rancé C, Módis K, et al. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol*. 2014;171:2099-2122.

14. Ezeño-Orbea J, Majtan T, Oyerant I, Kraus JP, Martínez-Cruz LA. Structural insight into the molecular mechanism of allostERIC activation of human cystathionine-ß-synthase by β-adenosylhomocysteine. *Proc Natl Acad Sci U S A*. 2014;111:E3845-E3852.

15. Singh S, Padavani D, Leslie RA, Chiku T, Banerjee R. Relative contributions of Cystathionine beta-synthase and gamma-cystathionase to H₂S biogenesis via alternative trans-sulfuration reactions. *J Biol Chem*. 2009;284:22457-22466.

16. Banerjee R. Catalytic promiscuity and heme-dependent redox regulation of H₂S synthesis. *Curr Opin Chem Biol*. 2017;37:115-121.

17. Kabl O, Yadav V, Banerjee R. Heme-dependent metabolite switching regulates H₂S synthesis in response to endoplasmic reticulum (ER) stress. *J Biol Chem*. 2016;291:16418-16423.

18. Yang J, Minkel P, Grove D, et al. Non-enzymatic hydrogen sulfide production from cystine in blood is catalyzed by iron and vitamin B6. *Commun Biol*. 2019;2:590-5914.

19. Berrazaga I, Micard V, Gueugneau M, Walrand S. The role of the anabolic properties of plant–versus animal-based protein sources in supporting muscle mass maintenance: a critical review. *Nutrients*. 2019;11:1825.

20. Zhubi-Bakjia F, Bajajkarti G, Bytyci I, et al. The impact of type of dietary protein, animal versus vegetable, in modifying cardiometabolic risk factors: a position paper from the International Lipid Expert Panel (ILEP). *Clin Nutr*. 2021;40:255-276.

21. Stipanuk MH, Ueki I, Dominy JE Jr, Summons CR, Hirschberger LL. Cystine dioxygenase: a robust system for regulation of cellular Cystine levels. *Amino Acids*. 2009;37:55-63.

22. Maclean KN, Jiang H, Aivazidis S, et al. Taurine treatment prevents derangement of the hepatic γ-glutamyl cycle and methylyglyoxal metabolism in a mouse model of classical homocysteinaemia: regulatory crosstalk between thiol and sulfenic acid metabolism. *FASEB J*. 2018;32:1265-1280.

23. Sun Q, Wang B, Li Y, et al. Taurine supplementation lowers blood pressure and improves vascular function in prehypertension randomized, double-blind, placebo-controlled study. *Hypertension*. 2016;67:541-549.

24. Singh S, Banerjee R. PLP-dependent H₂S biogenesis. *Biochim Biophys Acta*. 2011;1814:1518-1527.

25. Yin J, Ren W, Yang G, et al. L-Cysteine metabolism and its nutritional implications. *Mol Nutr Food Res*. 2016;60:134-146.

26. Ji Y, Kong X, Wang G, et al. Distribution and determinants of plasma homocysteine and total homocysteine in dialysis patients. *Am J Physiol Ren Physiol*. 2016;310:F947-F958.
33. Shinkai Y, Kumagai Y. Sulfane sulfur in toxicology: a novel defense system against electrophilic stress. *Toxicol Sci*. 2019;170:3-9.
34. Kimura H. Metabolic turnover of hydrogen sulfide. *Front Physiol.* 2012;3:101.
35. Hipólito A, Nunes SC, Vicente JB, Serpa J. Cysteine Aminotransferase (CAT): a pivotal sponsor in metabolic remodeling and an ally of 3-mercaptoppyruvate Sulfurtransferase (MST) in cancer. *Molecules*. 2020;25:3984.
36. Lu SC. Glutathione synthesis. *Biochem Biophys Acta*. 2013;1830:3143-3153.
37. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. *Proc Natl Acad Sci USA*. 2009;106:15424-15429.
38. Toohey JI. Possible involvement of hydrosulfide in B12-dependent Methyl group transfer. *Molecules*. 2017;22:582.
39. Crabtree MJ, Hale AB, Channon KM. Dihydrofolate reductase protects endothelial nitric oxide synthase from uncoupling in tetrahydrobiopterin deficiency. *Free Radic Biol Med*. 2011;50:1639-1646.
40. Chen P-H, Fu YS, Wang Y-M, Yang K-H, Wang DL, Huang B. Hydrogen sulfide increases nitric oxide production and subsequent S-nitrosylation in endothelial cells. *ScientificWorldJournal*. 2014;2014:480387.
41. Bucci M, Papapetropoulos A, Vellecco V, et al. Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity. *Arterioscler Thromb Vasc Biol*. 2010;30:1998-2004.
42. Zhang C-Y, Li XH, Zhang T, Fu J, Cui X-D. Hydrogen sulfide upregulates heme oxygenase-1 expression in rats with volume overload-induced heart failure. *Biomed Res Int*. 2013;1:454-458.
43. D’Araio E, Shaw N, Milward A, Demaine A, Whiteman M, Hodgkinson A. Hydrogen sulfide induces heme oxygenase-1 in human kidney cells. *Acta Diabetol*. 2014;51:155-157.
44. Matsui T, Sugiyama R, Sakashita K, et al. Hydrogen sulfide bypasses the rate-limiting oxygen activation of heme oxygenase. *J Biol Chem*. 2018;293:16931-16939.
45. Mitidieri E, Tramontano T, Gurgone D, et al. Mercaptopyruvate acts as endogenous vasodilator independently of 3-mercaptoppyruvate sulfurtransferase activity. *Nitric Oxide*. 2018;75:53-59.
46. Wójcik OP, Koenig KL, Zeleniuch-Jacquotte A, Costa M, Chen Y. The potential protective effects of taurine on coronary heart disease. *Atherosclerosis*. 2010;208:19-25.