Research paper

Divergent behavior of hydrogen sulfide pools and of the sulfur metabolite lanthionine, a novel uremic toxin, in dialysis patients

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Abstract
Dialysis patients display a high cardiovascular mortality, the causes of which are still not completely explained, but are related to uremic toxicity. Among uremic toxins, homocysteine and cysteine are both substrates of cystathionine β-synthase and cystathionine γ-lyase in hydrogen sulfide biosynthesis, leading to the formation of two sulfur metabolites, lanthionine and homolanthionine, considered stable indirect biomarkers of its production. Hydrogen sulfide is involved in the modulation of multiple pathophysiological responses. In uremia, we have demonstrated low plasma total hydrogen sulfide levels, due to reduced cystathionine γ-lyase expression.

Plasma hydrogen sulfide levels were measured in hemodialysis patients and healthy controls with three different techniques in comparison, allowing to discern the different pools of this gas. The protein-bound (the one thought to be the most active) and acid-labile forms are significantly decreased, while homolanthionine, but especially lanthionine, accumulate in the blood of uremic patients. The hemodialysis regimen plays a role in determining sulfur compounds levels, and lanthionine is partially removed by a single dialysis session.

Lanthionine inhibits hydrogen sulfide production in cell cultures under conditions comparable to in vivo ones. We therefore propose that lanthionine is a novel uremic toxin. The possible role of high lanthionine as a contributor to the genesis of hyperhomocysteinemia in uremia is discussed.

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1. Introduction

Chronic kidney disease (CKD) is characterized by a high cardiovascular (CV) mortality, which cannot be entirely explained by the increase in traditional CV risk factors. Among the numerous uremic toxins/biomarkers, retained in all stages, but more markedly in uremia, affecting the vasculature, sulfur-containing metabolites participating in the methionine-homocysteine cycle and the transsulfuration pathway, such as cysteine (Cys), and homocysteine (Hcy), are increased. Pertaining to these pathways is also hydrogen sulfide (H2S), the third gasotransmitter after nitric oxide and carbon monoxide, which exerts several beneficial biological functions (anti-oxidative and anti-inflammatory effects, lifespan extension, regulation of vascular tone and blood pressure-lowering, protection from ischemia-reperfusion injury, etc.) [1]. Low plasma H2S levels have been detected in uremia [2,3], which is linked to a downregulation of cystathionine-γ-lyase (CSE), one of the main H2S forming enzymes [4–7].

Cys and Hcy are utilized as substrates for H2S biosynthesis by the enzymes cystathionine-β-synthase (CBS), the above-mentioned CSE, and 3-mercaptopiruvate sulfurtransferase (MPST). Two novel sulfur metabolites, lanthionine and homolanthionine, have been found to be possible by-products of H2S production (Fig. 1) [8,9]. Condensation of two molecules of Cys (β-replacement reaction) produces H2S and lanthionine, while condensation of two molecules of Hcy produces H2S and homolanthionine (γ-replacement reaction).
reaction). Lanthionine and homolanthione are considered stable products of H\(_2\)S metabolism and indirect biomarkers of its production \[8,9\].

Accuracy and precision of H\(_2\)S detection in biological samples may be significantly affected by the various methodologies employed, which provide somewhat divergent results \[10,11\]. In fact, H\(_2\)S is present in different pools, which mediate various H\(_2\)S biological effects. Aim of our work was to establish whether the most important H\(_2\)S-related sulfur compounds displayed specific alterations, which could be the distinct signature of kidney failure. Furthermore, we intended to approach the possible mechanism(s) which, through unbalanced metabolism and/or deranged emunctory, may affect sulfur compounds levels, particularly those pertaining to increased CV risk in CKD. In our work, we tested three different methods (chromatographic with monobromobimane derivatization, potentiometric, utilizing a micro-electrode, and spectrophotometric with methylene blue) for plasma H\(_2\)S measurement in a control group and a group of hemodialysis patients, thus being able to compare results from all currently used methods and discern among the various H\(_2\)S forms in circulation. We also measured lanthionine and homolanthionine, as well as Hcy, Cys and other relevant compounds, in uremia compared to controls, and studied the effect of different hemodialysis modalities and those of a single dialysis session. In addition, the effects of lanthionine on H\(_2\)S release were also tested in cell cultures.

2. Materials and methods

2.1. Patients and study design

A control group of 22 healthy subjects recruited among hospital staff (mean age = 53 y, age range 32–59 y) and 67 uremic subjects (mean age = 63 y, age range 45–72 y) on chronic hemodialysis were recruited, provided that they were in stable clinical conditions, treated with dialysis since at least 6 months, Kt/V > 1.4, and not affected by systemic diseases such as lupus erythematosus, diabetes mellitus, cancer, or evidence of other systemic diseases antecedent to renal failure, which would be confounding variables. Patients were also hepatitis C virus antibody negative. Previous transplant patients were excluded. Patients were treated with erythropoietin and other drugs commonly utilized in this population for anemia, hypertension and secondary hyperparathyroidism, following KDOQI guidelines.

Patients were either on standard low-flux HD, with dialysis membrane polysulfone (n = 37, Fresenius Medical Care, Bad Homburg, Germany), or low-flux HD, with dialysis membrane polyethersulfone (n = 12, Nordic Medcom AB, Borås, Sweden), or HDF (n = 12), membrane high-flux steam-sterilized polysulfone, or PHF, with high-flux polyethersulfone (n = 6).

The group of uremic patients on standard low-flux HD with polyethersulfone or high-flux PHF was utilized also for studying H\(_2\)S behavior during dialysis (pre-post measurements and time-course), and sulfur compounds determinations.

All patients gave their informed consent. Procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by our institutions’ ethics committee (protocol number 374/2007 of the Second University of Naples).

Blood was drawn, in the fasting state, by venipuncture. In patients, blood was obtained before the session, or before and after the session, or at various times during the same session, as mentioned in the relevant experiments. Blood samples were drawn in plastic tubes, and plasma or serum were immediately obtained through brief (5’́) centrifugation at 3000 × g. Plasma or serum samples were aliquoted and stored at −20 °C before analysis.

2.2. H\(_2\)S detection with high performance liquid chromatography (HPLC) monobromobimane (MBB) method

Plasma was drawn utilizing lithium-heparin Vacutainers (BD Diagnostics 368884, Italy). Three different protocols were utilized in order to liberate and detect free H\(_2\)S, acid-labile sulfide, and bound sulfane sulfur (which are the major sulfide pools, important in regulating the amount of bioavailable sulfur) according to the general principle of Shen’s method \[10\], with modifications regarding one crucial step, as indicated below, in order to keep sample manipulation at a minimum. An Agilent 1100 series high
performance liquid chromatographer was used, equipped with an X Terra 18RP (5 μm, 80 Å, 4.6 × 250 mm) column (Phenomenex srl, Casalecchio di Reno, Italy), a fluorescence detector, and a gradient employing two mobile phases at 0.600 mL/min: (A) water 0.1% trifluoroacetic acid (v/v); (B) CH₃CN 99.9%, 0.1% TFA (v/v).

Free H₂S. In an hypoxic chamber, under nitrogen flux, 30 μL of plasma were mixed with 70 μL of reaction buffer (Tris–HCl 100 mM, pH 9.5, 0.1 mM Diethylentetriaminepentaacetic acid, DTPA), to release all H₂S present in samples and to stabilize H₂S in its HS⁻ form, and 50 μL of 10 mM MBG solution in CH₃CN. This mixture is incubated for 30 min in a hypoxic chamber at room temperature. After incubation, 50 μL of sulfosalicylic acid solution 200 mM were added and incubated on ice for 10 min, in order to stop the reaction and precipitate proteins. After incubation, samples are centrifuged at 16,000 × g at 4 °C for 10 min. The supernatant is injected into the HPLC system, and read at excitation 390 nm, emission 475 nm.

Acid-labile sulfide and bound sulfane sulfur. Acid-labile sulfide consists of sulfur present in the iron-sulfur clusters contained in iron-sulfur proteins (nonheme), while bound sulfane sulfur consists of H₂S bound to proteins (persulfides, polysulfides, etc.). We attempted to circumvent the problem linked to possible H₂S dispersion and preferred to omit needle removal of the solution by utilizing one-pot extraction. 50 μL of plasma were placed into a 4 mL Vacutainer without additives, and mixed with 450 μL of phosphate buffer 100 mM, pH 2.6, 0.1 mM DTPA to release H₂S from the acid-labile pool. Another 50 μL of plasma were then put in 4 mL Vacutainer without additives, and mixed with 450 μL di phosphate buffer 100 mM, pH 2.6, 0.1 mM DTPA, 1 mM tris(2-carboxyethyl) phosphine (TCEP), in order to release bound sulfane sulfur. Samples were then incubated for 30 min at room temperature on a rotating shaker. After incubation, 500 μL of reaction buffer (Tris–HCl 300 mM, pH 9.5, 0.1 mM DTPA) were added, and incubated again for 30 min, to trap the released H₂S from acid-labile and sulfane sulfur pools, and reach a conversion to HS⁻ which can then react with MBG to form sulfide dibimane, SDB. 50 μL of sulfosalicylic 200 mM solution were added to stop the reaction and precipitate proteins, and the mixture was incubated on ice for 10 min, then centrifuged at 16,000 × g at 4 °C for 10 min. The supernatant was used for HPLC separation and fluorescence detection. H₂S concentration was calculated utilizing a standard curve obtained with Na₂S standard solutions.

2.3. H₂S detection with methylene blue method (spectrophotometric)

Plasma was drawn utilizing EDTA Vacutainers (BD Diagnostics 368856, Italy). Samples were mixed with Zinc Acetate (to trap H₂S), N,N-dimethyl-p-phenylenediamine (40 mM in HCl 7.2 M) and FeCl₃ (30 mM in HCl 1.2 M) [12]. To deproteinize samples, 10% trichloroacetic acid solution (TCA) was added. The final product thus obtained (methylene blue) was read spectrophotometrically at 670 nm (Thermo SpectronicBiomet 3 UV/VIS Spectrophotometer). H₂S concentration of plasma samples was calculated against a standard curve obtained with NaHS.

2.4. H₂S detection with potentiometric method (ion-selective electrode)

Plasma was drawn utilizing lithium-heparin Vacutainers (BD Diagnostics 368884, Italy). Each sample was mixed with an antioxidant buffer (0.8 M sodium salicylate, 1.1 M NaOH, 0.2 M ascorbate) in 1:1 ratio and in the dark for 20 min at room temperature. After incubation, the potential was measured with a micro-electrode (Lazar Research Inc., Los Angeles CA, USA) and expressed in millivolts. The potential corresponds to the S²⁻ ion concentration, released under alkaline conditions from all H₂S present in the sample.

2.5. Plasma cysteine

Plasma from EDTA Vacutainers (BD Diagnostics 368856, Italy) was used. 10% tris-butylphosphate (TBP) in dimethyl-formamide (DMF) solution and 10% trichloroacetic acid solution in EDTA 1 mM were added to 300 μL of each sample and incubated at 4 °C for 30 min. After incubation, samples were centrifuged and the supernatant filtered and mixed with 20 μL of 1.55 M NaOH solution and 250 μL of 0.125 M borate buffer in 4 mM EDTA, and finally derivatized with 100 μL of 7-fluorobenzan-4-sulfonic acid (SBDF). This mix was incubated at 60 °C for 1 h 50 μL of derivatized samples were analyzed by HPLC with fluorescence detection. An X Terra 18RP (5 μm, 80 Å, 4.6 × 250 mm) column was used with an isocratic flow of 0.600 mL/min, mobile phase was KH₂PO₄ 0.1 M, pH 2.1, CH₃CN 4%, fluorescence detection (excitation 385 nm, emission 515 nm).

2.6. Plasma Hcy

Plasma from Citrate Vacutainer (BD Diagnostics 367704) was used. Total Hcy from plasma samples was measured with an automated latex enhanced immunoassay on IL Coagulation System (HemosIL, Instrumentation Laboratory SpA, Milano, Italy).

2.7. Lanthionine, homolanthionine, free Hcy, homoserine, cystathionine liquid chromatography tandem mass spectrometry (LC-MS/MS) measurements

Hcy, homoserine, lanthionine, and cystathionine were purchased from Sigma-Aldrich. All solutions and solvents were of the highest available purity and were suitable for LC–MS analysis. All stock solutions were stored at −20 °C.

Serum from Vacutainer SST II Advance (BD Diagnostics 367955, silica clotact/gel) of uremic patients and healthy volunteers was used. Samples (200 μL) were subject to simple protein precipitation with 600 μL ethanol and vortexed thoroughly. The mixture was stored at −20 °C for about 30 min to complete protein precipitation and then centrifuged at 13,000 × g for 10 min. Protein precipitation with ethanol (1:3 v/v) at −20 °C for about 30 min resulted in the highest yields for protein pellet and for all analytes. Extending the precipitation time beyond 30 min (i.e., up to 2 h) did not increase recovery. Moreover, this procedure was compatible with the mobile phase for further LC; thus, this solvent was subsequently used throughout the study. The supernatant was then directly transferred into HPLC auto sampler and 1 μL of supernatant was analyzed in a LC–MS/MS assay. The LC–MS/MS analyses were carried out by using a 6420 triple Q system with a HPLC 1100 series binary pump (Agilent, Waldbronn, Germany). The analytical column was a Phenomenex Kinetex 5μ 100 A C18. The mobile phase was generated by mixing eluent A (2% acetonitrile, 0.1% formic acid) and eluent B (CH₃CN 4%, 80 µL/h) in a 60:40 ratio.

MS analysis. All MRM transitions (Fig. 1, panel A) were optimized in a Turbo Ionspray (Agilent, Waldbronn, Germany) turbo ion spray source operated in positive 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 transitions (Fig. 1, panel A). The ideal conditions for detection were determined via Agilent MassHunter Optimizer software. Fig. 2 panel B shows the MRM chromatograms obtained for each target analyte showing a good selectivity for all analytes, with all transitions correctly occurring at the same retention time. Standard calibration curves 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. This method allows for the simultaneous measurement of serum free lanthionine, homolanthionine, free Hcy, homoserine, and cystathionine.

2.8. Cell cultures and reagents

The HepG2 cell line was purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. The cell line was grown in Dulbecco’s modified Eagle’s medium (GIBCO® DMEM, Invitrogen™, Carlsbad, CA) with 10% fetal bovine serum (FBS, GIBCO®) and 1% non-essential amino acids (Lonza BioWhittaker®). The cell culture medium was supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine. Cells were grown at 37 °C in a humidified atmosphere with 5% CO₂.

A modified methylene blue method assay was utilized, termed “agar trap method”, allowing accurate H₂S measurement in cell cultures without any H₂S loss [13]. The method utilizes a modified agar layer placed on the non-adherent surface of a cell flask (1% agar, 45 mM Zinc Acetate, 3 M NaOH). After solidification of the agar layer, cells were seeded and incubated overnight, then incubated with different H₂S-releasing compounds: L-cysteine (10 mM; Sigma 30089-100G), DL-Hcy (10 mM; Sigma H4628-1G), vitamin B₆ (6 mM; Benexol, Bayer), AdoMet (1 μM; Biolabs B9003S), DL-lanthionine (0.3 μM, the same concentration found in uremic serum; Sigma L8543). Parallel samples were treated with DL-Propargylglycine (PAG) 1 mM, a known CSE inhibitor, (Sigma P7888-250MG) as a negative control. H₂S, produced during 6 h and 24 h of incubation, was thus trapped in the agar layer, then released and quantified in situ by adding to each flask N,N-dimethyl-p-phenylenediamine sulfate (Sigma 186384; 40 mM in 7.2 M HCl) and FeCl₃ (Sigma 157740; 30 mM in 1.2 M HCl). After 20 min of incubation, the absorbance was read at 670 nm with Thermo Spectronic Biomate 3 UV/VIS Spectrophotometer. The concentration was calculated against a standard curve obtained with a NaHS solution (Sigma 161527) as H₂S donor, attained under the above-mentioned conditions. The cell monolayer was monitored during the

![Fig. 2. Multiple reaction monitoring (MRM) analysis: Panel A. MRM mass spectral parameters. Panel B. MRM chromatogram of the target compounds. Transitions and corresponding names are reported.](image)
experiments to confirm that the cells were normal and not rounded or detached due to unfavorable growth conditions. Further, cell viability was checked by PrestoBlue viability assay [14] to assess compound cytotoxicity.

2.9. Statistical analysis

Unpaired, paired, and one-sample Student’s t-test was utilized to compare means, as appropriate. Correlation tests (Pearson and Spearman) were used to assess linear associations between two variables. ONE-way ANOVA test for repeated measures was used in the dialysis time-course experiments. The results were analyzed with the statistics software GraphPad Prism Version 6.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Opposite behavior of circulating levels of H2S and relevant blood sulfur metabolites in uremic patients compared to those measured in healthy subjects

In Table 1, the main biochemistries of patients selected for this study are shown. These values are typical of those commonly present in uremic patients on hemodialysis.

Plasma H2S levels measured with the monobromobimane HPLC method were significantly decreased in uremic patients with respect to healthy controls (Fig. 3). In particular, while free H2S levels were not significantly different compared to controls (panel A), bound sulfane sulfur (consisting of H2S bound to proteins, such as persulfides, polysulfides, etc.), acid-labile sulfide (consisting of sulfur present in the iron-sulfur clusters contained in nonheme iron-sulfur proteins), and total H2S (panel B, C, D) were significantly reduced in uremic patients. Results, obtained with the potentiometric and spectrophotometric method, confirm that plasma H2S levels were significantly decreased in uremic patients (panel E, F).

Results clearly showed that HPLC, as expected, allowed to distinguish among different H2S fractions, while the other two methods were not selective with respect to the various H2S pools. In addition, it is clear that the three methods obtained different absolute plasma H2S values. In particular, HPLC provided much lower concentrations, compared with the other two methods. However, all methods concur in demonstrating lower levels in uremic patients, compared to control.

Conversely, circulating levels of the relevant sulfur metabolites were significantly elevated in uremic patients with respect to control subjects (Table 2), with the exception of homoserine, which was significantly decreased in uremic patients. Particularly paradoxical appeared the behavior of lanthionine and homolanthionine, which have been so far considered stable markers of H2S production. In fact, and somewhat surprisingly, lanthionine levels were increased by at least two orders of magnitude, in the patients compared to control, while homolanthionine levels doubled, in the face of generally decreased H2S pools.

Correlations between plasma H2S levels and homolanthionine and lanthionine in uremic patients were also assessed. In uremic patients, there were no significant correlations between lanthionine and plasma H2S levels considering all its measured forms. However, there was a significant positive correlation between homolanthionine and total H2S and acid-labile sulfide (respectively, \( r = 0.3673, p < 0.01 \); and \( r = 0.2977, p < 0.05 \)), but not between homolanthionine and bound sulfane sulfur and free H2S.

3.2. Basal plasma H2S pools differ in patients treated with various types of dialysis

Data were stratified according to the type of dialysis: hemodialysis (HD) low-flux polysulphone, HD low-flux polyethersulfone, hemodiafiltration (HDF) high-flux polysulphone, paired hemodiafiltration (PHF) high-flux polyethersulfone. Results are shown in Table 2.

The free form was significantly higher in the polysulphone-treated patients, while it was lower with polyethersulfone. The bound sulfane sulfur was lower in PHF patients, and higher in HDF. The acid-labile form did not differ. Total levels measured with HPLC were higher in HD and HDF and lower in PHF patients. These differences are somewhat mirrored by the levels obtained with the ion-selective electrode method, although the latter provided much higher concentration values, as noted above. Conversely, with the methylene blue method, levels were higher in patients treated with polyethersulfone.

3.3. Plasma H2S and sulfur metabolites circulating concentrations before and after a single dialysis session

For this study, results obtained in HD low-flux polysulphone and PHF high-flux polyethersulfone dialysis patients were merged. Free H2S didn’t change after a single dialysis session, bound sulfane sulfur increased but not significantly, while acid-labile sulfide and total H2S decreased significantly after dialysis. Plasma H2S levels measured with the potentiometric method increased significantly after dialysis; however, H2S measured with the spectrophotometric method (methylene blue method) didn’t show significant differences after dialysis (Table 4).

Sulfur metabolites levels decreased significantly after dialysis, with the exception of free Hcy, which was not different, and homoserine and homolanthionine, which decreased, but not significantly (Table 5).

Then the two patient groups (HD low-flux polysulphone and PHF high-flux polyethersulfone) were considered separately, observing the following.

Free H2S increased significantly after PHF, but not after HD (Fig. 4, panel A). Bound sulfane sulfur increased significantly after PHF, but not after HD (panel B); levels were significantly lower in PHF at the start of dialysis with respect to HD. Acid-labile sulfide decreased significantly after HD, but not after PHF (panel C). Total H2S decreased significantly after HD, but not after PHF (panel D). H2S measured with the potentiometric method increased significantly after HD dialysis and increased, but not significantly, after PHF dialysis (panel E), while H2S measured with spectrophotometric method didn’t show any significant variations after HD and PHF dialysis (panel F).

Both types of dialysis were able to significantly remove the sulfur metabolites of interest (Fig. 5), although to different

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patients</th>
</tr>
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<tbody>
<tr>
<td>Creatinine (mg/dL) (0.5–1.1)</td>
<td>8.56 ± 0.3</td>
</tr>
<tr>
<td>Urea (mg/dL) (12–50)</td>
<td>124.1 ± 5.6</td>
</tr>
<tr>
<td>Calcium (mg/dL) (8.6–10.5)</td>
<td>9.03 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus (mg/dL) (2.4–4.1)</td>
<td>4.75 ± 0.2</td>
</tr>
<tr>
<td>Parathyroid hormone (pg/mL) (12–72)</td>
<td>320.8 ± 58.96</td>
</tr>
<tr>
<td>C Reactive Protein (mg/L) (&lt;5)</td>
<td>7.08 ± 2.3</td>
</tr>
<tr>
<td>Albumin (g/dL) (3.5–5.2)</td>
<td>3.86 ± 0.10</td>
</tr>
<tr>
<td>Mean Corpuscular Volume (FL) (85–98)</td>
<td>95.36 ± 1.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL) (13.5–17)</td>
<td>11.58 ± 0.2</td>
</tr>
<tr>
<td>Potassium (mmol/L) (3.6–5.0)</td>
<td>5.5 ± 0.09</td>
</tr>
<tr>
<td>Alkaline Phosphatase (U/L) (30–120)</td>
<td>85.6 ± 5.10</td>
</tr>
</tbody>
</table>
levels of significance, with the exception of free Hcy (panel C). It can also be noted that basal levels (the pre-dialysis values) of sulfur metabolites in PHF were lower than those present in HD (Fig. 5).

Plasma H₂S levels during a single dialysis session (time course) were measured (Fig. 6). Results showed that bound sulfane sulfur levels increase significantly during PHF (panel D), but not in HD (panel C), while acid-labile sulfide decreased significantly during HD (panel E), but not in PHF (panel F). Interestingly, variations started from the initial stages of the dialysis session and changed gradually. Total and free H₂S, in both types of dialysis, did not vary significantly (panel A, B, G, H).

H₂S measured with potentiometric method did not show any significant variation during HD and PHF dialysis; also H₂S measured with spectrophotometric method did not show any significant variation during HD and PHF dialysis (not shown).

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**Figure 3.** H₂S measured in its various forms with the MBB method (HPLC, panels A-D), potentiometric method (ion-selective electrode, panel E), spectrophotometric method (methylene blue method, panel F), (mean ± SEM). Controls (white bar); patients (black bar). *p < 0.05, **p < 0.01, ***p < 0.001.

**Table 2**

Values of the various relevant sulfur metabolites (mean ± SEM).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy (µM)</td>
<td>13.50 ± 2.6</td>
<td>35.95 ± 2.5***</td>
</tr>
<tr>
<td>Cys (µM)</td>
<td>76.44 ± 2.7</td>
<td>145.3 ± 7.1***</td>
</tr>
<tr>
<td>Free Hcy (µM)</td>
<td>0.68 ± 0.08</td>
<td>1.29 ± 0.09***</td>
</tr>
<tr>
<td>Cystathionine (µM)</td>
<td>&lt;0.002*</td>
<td>5.42 ± 0.5***</td>
</tr>
<tr>
<td>Homoserine (µM)</td>
<td>6.13 ± 0.2</td>
<td>5.53 ± 0.3*</td>
</tr>
<tr>
<td>Lanthionine (µM)</td>
<td>&lt;0.002*</td>
<td>0.32 ± 0.03***</td>
</tr>
<tr>
<td>Homolanthionine (µM)</td>
<td>0.26 ± 0.04</td>
<td>0.52 ± 0.1*</td>
</tr>
</tbody>
</table>

* Detection limit.

"p < 0.05,**p < 0.01, ***p < 0.001.
treatment and in the presence of the indicated substrates and generally higher than that produced after 6 h (study H2S production, and the regulatory properties of the major HepG2 appear to be one of the most appropriate cell systems to metabolism including the transsulfuration pathway. Therefore, expresses all the enzymatic machinery for sulfur amino acid cells during 6 h (study, utilizing the agar trap assay system.

Although no data is available about the possible effects of this compound had been shown to specifically inhibit CBS [15], although less pronounced, after 24 h of incubation (Fig. 7).

Traditional risk factors and those linked to the severe loss chronic diseases such as ischemia-reperfusion injury, pulmonary hypertension, atherosclerosis, and notably CKD progression and disease. Traditional risk factors and those linked to the severe loss complications [1,17].

Among the various sulfur metabolites altered in uremic patients on hemodialysis, we focused particularly on lanthionine, since this compound had been shown to specifically inhibit CBS [15], although no data is available about the possible effects of this interference on H2S production. Hepatocarcinoma cell line (HepG2) expresses all the enzymatic machinery for sulfur amino acid metabolism including the transsulfuration pathway. Therefore, HepG2 appear to be one of the most appropriate cell systems to study H2S production, and the regulatory properties of the major enzymes involved, CBS and CSE. HepG2 cells were incubated with various substrates and cofactors for H2S production (Hcy, Cys, S-adenosylmethionine, etc.) with or without lanthionine, at concentrations comparable to those measured in the uremic serum in this study, utilizing the agar trap assay system.

Results in Fig. 7 show the amount of H2S produced by HepG2 cells during 6 h (panel A), and 24 h (panel B), upon lanthionine treatment and in the presence of the indicated substrates and cofactors.

The total amount of H2S produced after 24 h of incubation was generally higher than that produced after 6 h (panel B vs panel A). H2S release in cells incubated with Hcy, Cys, S-adenosylmethionine (AdoMet) and vitamin B6 was significantly higher compared to control cells. This increase was significant at 6 h and could still be detected after 24 h of incubation (compare panel A and B in Fig. 6).

Pre-treatment with lanthionine decreased significantly the amount of H2S produced by cells during 6 h incubation under conditions of maximal H2S release (Fig. 7, panel A). This effect was still significant, although less pronounced, after 24 h of incubation (Fig. 7, panel B). The extent of lanthionine inhibition of H2S release was comparable to the effect of DL-propargylglycine (DL-PAG), a known transsulfuration inhibitor, observed at both 6 and 24 h incubation times (Fig. 7). Importantly, the inhibitory effect of lanthionine on H2S release was nearly abolished when samples were incubated in the absence of AdoMet, both at 6 and 24 h (Fig. 7).

These results demonstrated that increased lanthionine, as the result of CKD, can effectively reduce H2S production in the liver, thus establishing an altered metabolic crosstalk between these two organs.

### 3.4. Lanthionine inhibits H2S production by interfering with CBS activity

Among the various sulfur metabolites altered in uremic patients on hemodialysis, we focused particularly on lanthionine, since this compound had been shown to specifically inhibit CBS [15], although no data is available about the possible effects of this interference on H2S production. Hepatocarcinoma cell line (HepG2) expresses all the enzymatic machinery for sulfur amino acid metabolism including the transsulfuration pathway. Therefore, HepG2 appear to be one of the most appropriate cell systems to study H2S production, and the regulatory properties of the major enzymes involved, CBS and CSE. HepG2 cells were incubated with various substrates and cofactors for H2S production (Hcy, Cys, S-adenosylmethionine, etc.) with or without lanthionine, at concentrations comparable to those measured in the uremic serum in this study, utilizing the agar trap assay system.

Results in Fig. 7 show the amount of H2S produced by HepG2 cells during 6 h (panel A), and 24 h (panel B), upon lanthionine treatment and in the presence of the indicated substrates and cofactors.

The total amount of H2S produced after 24 h of incubation was generally higher than that produced after 6 h (panel B vs panel A). H2S release in cells incubated with Hcy, Cys, S-adenosylmethionine (AdoMet) and vitamin B6 was significantly higher compared to control cells. This increase was significant at 6 h and could still be detected after 24 h of incubation (compare panel A and B in Fig. 6).

Pre-treatment with lanthionine decreased significantly the amount of H2S produced by cells during 6 h incubation under conditions of maximal H2S release (Fig. 7, panel A). This effect was still significant, although less pronounced, after 24 h of incubation (Fig. 7, panel B). The extent of lanthionine inhibition of H2S release was comparable to the effect of DL-propargylglycine (DL-PAG), a known transsulfuration inhibitor, observed at both 6 and 24 h incubation times (Fig. 7). Importantly, the inhibitory effect of lanthionine on H2S release was nearly abolished when samples were incubated in the absence of AdoMet, both at 6 and 24 h (Fig. 7).

These results demonstrated that increased lanthionine, as the result of CKD, can effectively reduce H2S production in the liver, thus establishing an altered metabolic crosstalk between these two organs.

### 4. Discussion

CKD patients, including those undergoing maintenance dialysis, have a disproportionately high morbidity and mortality due to CV disease. Traditional risk factors and those linked to the severe loss of kidney function cannot entirely explain this CV risk increase [16].

H2S is involved in modulation of various physiological responses. In general, it acts as a protective agent in oxygenation, inflammation, and apoptosis, all events paving the way to acute and chronic diseases such as ischemia-reperfusion injury, pulmonary hypertension, atherosclerosis, and notably CKD progression and complications [1,17].

Plasma H2S levels, measured spectrophotometrically, are reduced in hemodialysis patients with respect to healthy controls [2,18]. This reduction was attributed to the down-regulation of CSE expression [2,19]. Aminzadeh and Vaziri also demonstrated low H2S levels in a rat model of CKD; the alteration is linked to CKD progression [3].

The best method for H2S measurement has caused considerable

Table 3

<table>
<thead>
<tr>
<th>H2S (µM)</th>
<th>HD low-flux polysulfone</th>
<th>HD low-flux polyethersulfone</th>
<th>HDF high-flux polysulfone</th>
<th>PHF high-flux polyethersulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monobromobimamide method, HPLC (Free)</td>
<td>8.16 ± 0.2***</td>
<td>4.04 ± 0.4</td>
<td>8.23 ± 0.6</td>
<td>3.40 ± 0.5***</td>
</tr>
<tr>
<td>Monobromobimamide method, HPLC (bound sulfane sulfur)</td>
<td>2.03 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>3.02 ± 0.6*</td>
<td>1.36 ± 0.3</td>
</tr>
<tr>
<td>Monobromobimamide method, HPLC (Acid-labile sulfide)</td>
<td>4.11 ± 0.4</td>
<td>5.57 ± 0.4</td>
<td>4.50 ± 0.9</td>
<td>5.82 ± 0.6</td>
</tr>
<tr>
<td>Ion-selective electrode</td>
<td>14.06 ± 0.5***</td>
<td>11.85 ± 0.7</td>
<td>16.10 ± 1.5***</td>
<td>10.60 ± 0.9</td>
</tr>
<tr>
<td>Methylen blue method</td>
<td>75.32 ± 2.1***</td>
<td>58.68 ± 8.6</td>
<td>74.39 ± 2.8</td>
<td>53.87 ± 8.1***</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001.

Table 4

<table>
<thead>
<tr>
<th>H2S (µM)</th>
<th>PRE</th>
<th>POST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monobromobimamide method, HPLC (Free)</td>
<td>3.83 ± 0.3</td>
<td>3.80 ± 0.3</td>
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<tr>
<td>Monobromobimamide method, HPLC (bound sulfane sulfur)</td>
<td>1.49 ± 0.2</td>
<td>1.57 ± 0.2</td>
</tr>
<tr>
<td>Monobromobimamide method, HPLC (Acid-labile sulfide)</td>
<td>5.74 ± 0.3</td>
<td>4.44 ± 0.3***</td>
</tr>
<tr>
<td>Monobromobimamide method, HPLC (Total)</td>
<td>11.45 ± 0.6</td>
<td>9.6 ± 0.5***</td>
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<tr>
<td>Ion-selective electrode</td>
<td>73.21 ± 9.9</td>
<td>79.86 ± 10.0***</td>
</tr>
<tr>
<td>Methylen blue method</td>
<td>34.16 ± 3.5</td>
<td>29.75 ± 3.9</td>
</tr>
</tbody>
</table>

*p < 0.05, **p ≤ 0.01, ***p < 0.001.

Table 5

<table>
<thead>
<tr>
<th>Hcy (µM)</th>
<th>Cys (µM)</th>
<th>Free Hcy (µM)</th>
<th>Cystathionine (µM)</th>
<th>Homocysteine (µM)</th>
<th>Lanthionine (µM)</th>
<th>Homolanthionine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.98 ± 6.78</td>
<td>139.2 ± 13.73</td>
<td>1.59 ± 0.4</td>
<td>6.83 ± 1.6</td>
<td>5.45 ± 0.8</td>
<td>0.34 ± 0.06</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>26.48 ± 5.6***</td>
<td>64.31 ± 6.8***</td>
<td>1.44 ± 0.3</td>
<td>1.59 ± 0.5**</td>
<td>4.03 ± 0.3</td>
<td>0.15 ± 0.03**</td>
<td>0.13 ± 0.05</td>
</tr>
</tbody>
</table>

*p ≤ 0.01, ***p < 0.001.
controversy. H$_2$S exists in various chemical forms: free (hydro-sulphide anion, HS$^-$), acid-labile (bound to the sulfur-iron clusters of proteins), bound sulfane sulfur (bound to proteins, i.e. poly-sulfides, persulfides, etc.) [10,19]. Total H$_2$S refers to all these forms together. Total H$_2$S levels can be measured with different methods, as mentioned, among which the most commonly used is the methylene blue method with spectrophotometric detection. However, this method allows to quantify free H$_2$S, although, due to acidic sample treatment, acid-labile sulfide can be also freed to some extent, thus possibly and unpredictably decreasing overall accuracy. The potentiometric method, utilizing a micro-electrode, measures the S$_2$O$_3^-$ ion directly. It actually allows to quantify the free form, plus the bound sulfane sulfur, by mixing the sample with an antioxidant buffer (a strong alkaline solution). Consequently, results provided this method may be inaccurate, and/or difficult to interpret. The most selective and accurate method is given by HPLC separation and detection, because the sample is derivatized with monobromobimane, and, by using a hypoxic chamber, there is no H$_2$S dispersion. In addition, all H$_2$S forms can be measured separately. In this article, all three methods were utilized and compared.

In our work, plasma H$_2$S levels are decreased in uremic patients with respect to healthy controls, only partly in line with previous results. In fact, this reduction is confined to the acid-labile sulfide, bound sulfane sulfur forms, and total levels, as evidenced by results obtained with the HPLC method, while free levels did not vary significantly.

Key enzymes in H$_2$S production are CBS and CSE. These enzymes utilize as substrates Cys and Hcy, whose levels are both increased in uremic patients. Two of the various H$_2$S generating reactions, i.e. Cys $\beta$-replacement and Hcy $\gamma$-replacement, yield lanthionine or homolanthionine [8], two novel sulfur metabolites, which have been proposed to be useful for monitoring H$_2$S production.

Our results show that, like all the other sulfur metabolites, lanthionine and homolanthionine are increased in uremic patients...
with respect to control subjects, despite the above mentioned opposite modifications in H2S concentrations. A major difference is seen regarding lanthionine, which increases by about two orders of magnitude, while homolanthionine doubles.

Lanthionine is involved in CBS inhibition [15], and our results showed that this inhibition determines a significant impairment of H2S production in cell cultures. Then, as well, it can be hypothesized that the increase in blood levels of this compound found in our patients could contribute at least in part to the degree of hyperhomocysteinemia in uremia, thus providing a mechanism in

![Figure 5](image_url)

Fig. 5. Concentrations of the various sulfur metabolites, before (PRE) and after dialysis (POST) in HD polyethersulfone and PHF (mean ± SEM). PRE HD (white bar), POST HD (vertical line bar), PRE PHF (black bar), POST PHF (cross-hatched bar). *p < 0.05, **p ≤ 0.01.
Fig. 6. Time course of the various forms of H₂S, in a single dialysis session, in HD polyethersulfone and PHF (mean & whiskers min to max). *p < 0.05 **p < 0.01.

Fig. 7. Amount of H₂S produced by HepG2 cells incubated with various substrates. Panel A) 6 h of incubation; panel B) 24 h of incubation (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001. White bar = Control. Black bar = DL-Hcy 10 mM + L-Cys 10 mM + AdoMet 1 μM + vitamin B₆ 6 mM. Horizontal line bar = Pre-incubation of 2 h with DL-lanthionine 0.3 μM and incubation with DL-Hcy 10 mM + L-Cys 10 mM + AdoMet 1 μM + vitamin B₆ 6 mM. Checkered bar = DL-Hcy 10 mM + L-Cys 10 mM + vitamin B₆ 6 mM. Vertical line bar = Pre-incubation of 2 h with DL-lanthionine 0.3 μM and incubation with DL-Hcy 10 mM + L-Cys 10 mM + AdoMet 1 μM + vitamin B₆ 6 mM. Cross-hatched bar = Pre-incubation of 2 h with DL-PAG 1 mM and incubation with DL-Hcy 10 mM + L-Cys 10 mM + AdoMet 1 μM + vitamin B₆ 6 mM.
the pathophysiology of this derangement, which is still not completely understood. It has been in fact suggested that hyperhomocysteinemia in uremia may be due to inhibition, by an unknown uremic toxin, of Hcy-metabolizing enzymes, which is supported by the finding of reduced CSE transcription in mononuclear cells in vivo in uremic patients [2].

Our present data showed rather high circulating levels of the amino acid homoserine in the normal; these levels are decreased in uremia and are further lowered by dialysis, indicating that this is not a substantial retention product. Homoserine has been proposed as an alternative major product of H2S formation from Hcy, by α, γ-elimination reaction carried out by CSE [8]. Therefore, we can suggest that homoserine is a more likely indicator of reduced H2S production in uremia, compared to homocysteine and lanthionine.

The type of dialysis influences the levels of the different forms of H2S. Comparing the four different types of dialysis (low-flux HD with polysulfonie and polyethersulfone, and high-flux HDF with polysulfone and PHF with polyethersulfone), it emerges that the bound form and accordingly the total H2S levels are increased in high-flux dialysis with polysulfone. The other methods give somewhat different results, perhaps mirroring the different moieties to which the specific method is sensitive. Considering the effects of a single session, the acid labile form is dialyzed, while the bound forms tends toward an increase, as also indicated by the significant increase seen with the ion selective method. If the two types of dialysis are analyzed separately, in PHF, the bound form increases, although the patients start with lower levels, and all sulfur compounds taken as a whole are dialyzed better, while with HD with polyethersulfone all H2S forms are removed, as well as the sulfur compounds of interest.

We previously reported on the ability of a single hemodialysis session to increase plasma H2S [17]. However, these patients were not well characterized with respect to B vitamin status, in particular folate levels, or type of dialysis or filter utilized, or other dialysis parameters, such as dialysis time, vintage, Kt/V, etc. In the present study, only PHF patients showed a non-significant increase in total levels, and a significant increase in bound sulfane sulfur.

Homocysteine and especially lanthionine are removed rather efficiently by dialysis, in that their levels are reduced by half (better with PHF), although far from being normalized, particularly in the case of lanthionine. In the time-course experiments, PHF treatment induces an increase in the bound sulfane sulfur form, which is perhaps a remarkable feature, in consideration of the functional meaning of this H2S pool. Changes occur during the whole dialysis session, without any abrupt modification, that is they start from the beginning of treatment, suggesting that these variations are due to dialysis and not to other dialysis-related factors (e.g. hypotension at the end of dialysis session). All in all, the high-flux techniques seem to perform better compared to low-flux, in terms of respecting the bound H2S form concentration, and in terms of lanthionine removal.

5. Conclusions

This work underlines the value of measuring H2S in its various forms, because it emerged that, in uremia, prevalently it is the bound form which is affected. The sulfur compound lanthionine, far from being a marker of H2S production, emerges as novel uremic retention product, recommending cautious assessment of parameters of renal function before assuming that homocysteine or homolanthionine levels may adequately monitor H2S production in vivo. Lanthionine accumulation is able to hamper H2S production and could contribute, at least in part, to the development of hyperhomocysteinemia in uremia, therefore representing a new uremic toxin.

Disclosure

All the authors declared no competing interests.

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