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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.

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 (H_2S), 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 H_2S levels have been detected in uremia [2,3], which is linked to a downregulation of cystathionine-ɣ-lyase (CSE), one of the main H_2S forming enzymes [4–7].

Cys and Hcy are utilized as substrates for H_2S 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 H_2S production (Fig. 1) [8,9]. Condensation of two molecules of Cys (β-replacement reaction) produces H_2S and lanthionine, while condensation of two molecules of Hcy produces H_2S and homolanthionine (γ-replacement

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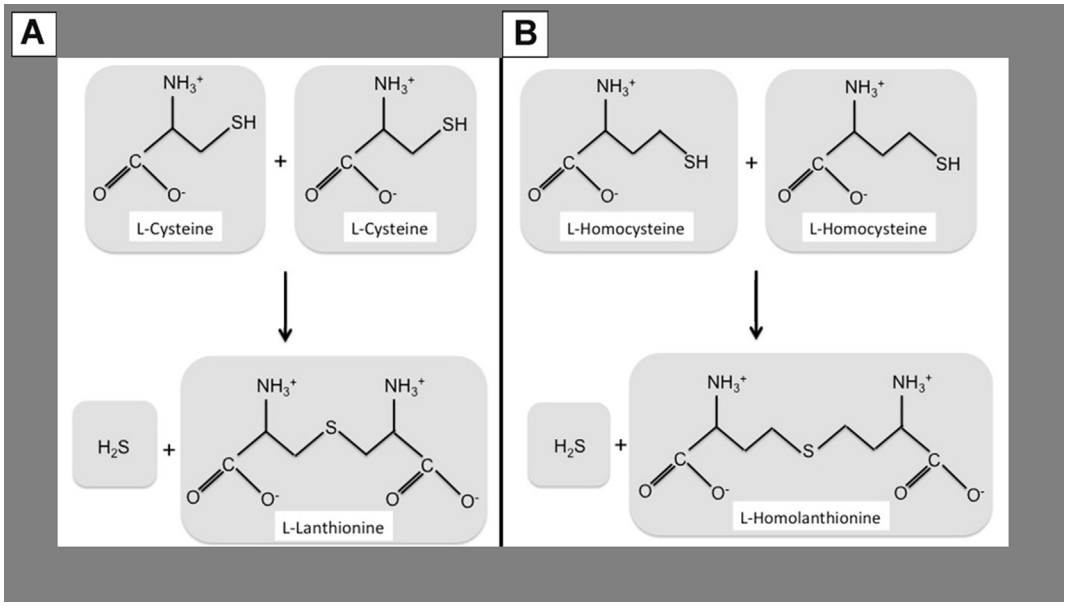

Fig. 1. Lanthionine and homolanthionine formation. Lanthionine is formed from condensation of two Cys molecules (panel A). Homolanthionine is formed from condensation of two Hcy molecules (panel B). Both reactions can be carried out by either CBS or CSE independently, under physiological conditions.

reaction). Lanthionine and homolanthione are considered stable products of H_2S metabolism and indirect biomarkers of its production [8,9].

Accuracy and precision of H_2S detection in biological samples may be significantly affected by the various methodologies employed, which provide somewhat divergent results [10,11]. In fact, H_2S is present in different pools, which mediate various H_2S biological effects. Aim of our work was to establish whether the most important H_2S-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_2S 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_2S 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_2S 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_2S 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_2S 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_2S, 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 XTerra 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% TFA (trifluoroacetic acid) (v/v); (B) CH_3CN 99.9%, 0.1% TFA (v/v).

Free H_2S. 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 Diethylenetriaminepentaacetic acid, DTPA), to release all H_2S present in samples and to stabilize H_2S in its HS^- form, and 50 μL of 10 mM MBB solution in CH_3CN. This mixture is incubated for 30 min in a hypoxic chamber at room temperature. After incubation, 50 μL of sulfosalicilic 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 H_2S bound to proteins (persulfides, polysulfides, etc). We attempted to circumvent the problem linked to possible H_2S 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 H_2S 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 H_2S from acid-labile and sulfane sulfur pools, and reach a conversion to HS^- which can then react with MBB to form sulfide dibimane, SDB. 50 μL of sulfosalicilic 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 16000 × g at 4 °C for 10 min. The supernatant was used for HPLC separation and fluorescence detection. H_2S concentration was calculated utilizing a standard curve obtained with Na_2S standard solutions.

2.3. H_2S detection with methylene blue method (spectrophotometric)

Plasma was drawn utilizing EDTA Vacutainers (BD Diagnostics 368856, Italy). Samples were mixed with Zinc Acetate (to trap H_2S), *N*,*N*-dimethyl-*p*-phenylenediamine (40 mM in HCl 7.2 M) and $FeCl_3$ (30 mM in HCl 1.2 M) [12]. To deproteinize samples, 10% trichloracetic acid solution (TCA) was added. The final product thus obtained (methylene blue) was read spectrophotometrically at 670 nm (Thermo SpectronicBiomate 3 UV/VIS Spectrophotometer). H_2S concentration of plasma samples was calculated against a standard curve obtained with NaHS.

2.4. H_2S 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 microelectrode (Lazar Research Inc., Los Angeles CA, USA) and expressed in millivolts. The potential corresponds to the S^{2-} ion concentration, released under alkaline conditions from all H_2S present in the sample.

2.5. Plasma cysteine

Plasma from EDTA Vacutainers (BD Diagnostics 368856, Italy) was used. 10% tris-butylphosphine (TBF) in dimethyl-formamide (DMF) solution and 10% trichloracetic 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 XTerra 18RP (5 μm, 80 Å, 4.6 × 250 mm) column was used with an isocratic flow of 0.600 mL/min, mobile phase was KH_2PO_4 0.1 M, pH 2.1, CH_3CN 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 5u 100 A C18. The mobile phase was generated by mixing eluent A (2% acetonitrile, 0.1% formic acid) and eluent B (95% acetonitrile and 0.1% formic acid), flow rate 0.200 mL/min. Starting condition was 5%–95% eluent A in 8 min, then brought to 100% in 2 min. Tandem mass spectrometry was performed using a 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\uL 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

A

Compound Name	Precursor Ion m/z	Product Ion m/z	Dwell (ms)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Polarity	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification	Linear range (pg/ul)
cystathionine	223,3	134,1	200	81	5	7	Positive	0,54	1,62	0,5-150
cystathionine	223,3	88	200	81	25	7	Positive	0,54	1,62	0,5-150
Lanthionine	209,31	120	200	81	9	7	Positive	0,38	1,15	0,5-150
Lanthionine	209,31	74	200	81	25	7	Positive	0,38	1,15	0,5-150
homocysteine	136,19	90	200	81	5	7	Positive	0,31	0,93	0,5-150
homocysteine	136,19	56,1	200	81	17	7	Positive	0,31	0,93	0,5-150
homoserine	120,11	74	200	81	5	7	Positive	0,14	0,43	0,5-150
homoserine	120,11	56,1	200	81	17	7	Positive	0,14	0,43	0,5-150
homolanthionine	237	148	200	81	5	7	Positive	0,42	1,07	0,5-150
homolanthionine	237	102	200	81	30	7	Positive	0,42	1,07	0,5-150

B

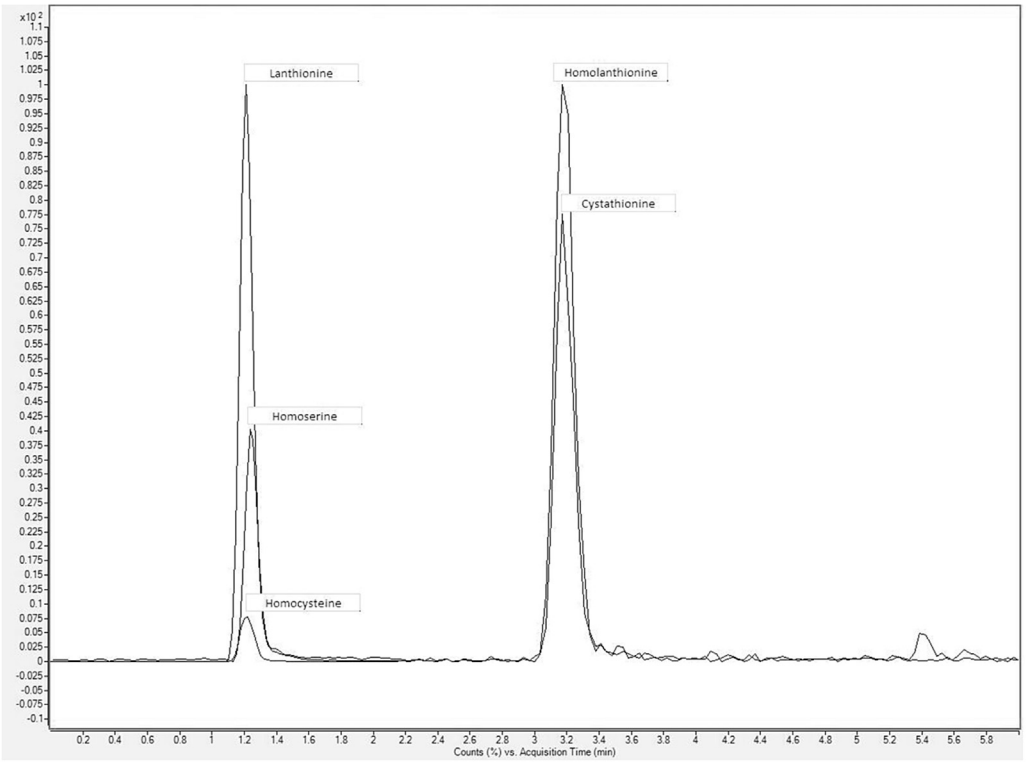

Fig. 2. Multiple reaction monitoring (MRM) analysis: Panel A. MRM mass spectral parameters. Panel B. MRM chromatogram of the target compounds. Transitions and corresponding names are reported.

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% CO_2.

A modified methylene blue method assay was utilized, termed "agar trap method", allowing accurate H_2S measurement in cell cultures without any H_2S 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 H_2S-releasing compounds: L-cysteine (10 mM; Sigma 30089-100G), DL-Hcy (10 mM; Sigma H4628-1G), vitamin B_6 (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. H_2S, 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 $FeCl_3$ (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 H_2S donor, attained under the above-mentioned conditions. The cell monolayer was monitored during the

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 H_2S 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 H_2S levels measured with the monobromobimane HPLC method were significantly decreased in uremic patients with respect to healthy controls (Fig. 3). In particular, while free H_2S levels were not significantly different compared to controls (**panel A**), bound sulfane sulfur (consisting of H_2S 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 H_2S (**panel B, C, D**) were significantly reduced in uremic patients. Results, obtained with the potentiometric and spectrophotometric method, confirm that plasma H_2S levels were significantly decreased in uremic patients (**panel E, F**).

Results clearly showed that HPLC, as expected, allowed to distinguish among different H_2S fractions, while the other two methods were not selective with respect to the various H_2S pools. In addition, it is clear that the three methods obtained different absolute plasma H_2S 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 H_2S 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 H_2S pools.

Correlations between plasma H_2S levels and homolanthionine and lanthionine in uremic patients were also assessed. In uremic patients, there were no significant correlations between lanthionine and plasma H_2S levels considering all its measured forms. However, there was a significant positive correlation between homolanthionine and total H_2S 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 H_2S.

Table 1
Main biochemical parameters (in parentheses units and normal laboratory values) of uremic patients (mean ± standard error of mean, SEM).

	Patients
Creatinine (mg/dL) (0.5–1.1)	8.56 ± 0.3
Urea (mg/dL) (12–50)	124.1 ± 5.6
Calcium (mg/dL) (8.6–10.5)	9.03 ± 0.1
Phosphorus (mg/dL) (2.4–4.1)	4.75 ± 0.2
Parathyroid hormone (pg/mL) (12–72)	320.8 ± 58.96
C Reactive Protein (mg/L) (<5)	7.08 ± 2.3
Albumin (g/dL) (3.5–5.2)	3.86 ± 0.10
Mean Corpuscolar Volume (fL) (85–98)	95.36 ± 1.6
Hemoglobin (g/dL) (13.5–17)	11.58 ± 0.2
Potassium (mmol/L) (3.6–5.0)	5.5 ± 0.09
Alkaline Phosphatase (U/L) (30–120)	85.6 ± 5.10

3.2. Basal plasma H_2S pools differ in patients treated with various types of dialysis

Data were stratified according to the type of dialysis: hemodialysis (HD) low-flux polysulfone, HD low-flux polyethersulfone, hemodiafiltration (HDF) high-flux polysulfone, paired hemodiafiltration (PHF) high-flux polyethersulfone. Results are shown in Table 3.

The free form was significantly higher in the polysulphone-treated patients, while it was lower with polyethersulphone. 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 polyethersulphone.

3.3. Plasma H_2S and sulfur metabolites circulating concentrations before and after a single dialysis session

For this study, results obtained in HD low-flux polyethersulfone and PHF high-flux polyethersulfone dialysis patients were merged. Free H_2S didn't change after a single dialysis session, bound sulfane sulfur increased but not significantly, while acid-labile sulfide and total H_2S decreased significantly after dialysis. Plasma H_2S levels measured with the potentiometric method increased significantly after dialysis; however, H_2S 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 polyethersulfone and PHF high-flux polyethersulfone) were considered separately, observing the following.

Free H_2S 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 H_2S decreased significantly after HD, but not after PHF (**panel D**). H_2S measured with the potentiometric method increased significantly after HD dialysis and increased, but not significantly, after PHF dialysis (**panel E**), while H_2S measured with spectrophotometric method didn't shown 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

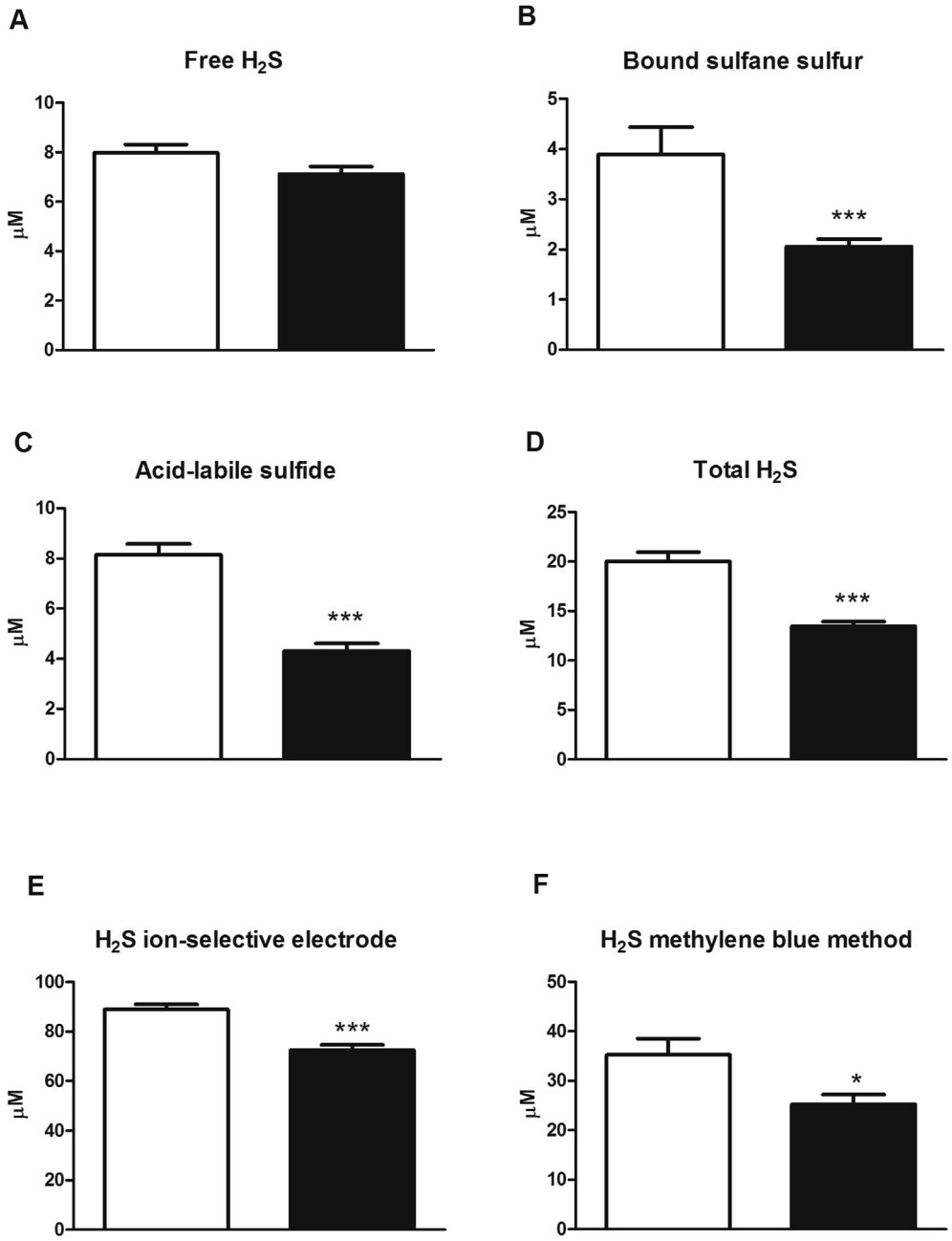

Fig. 3. H_2S measured in its various forms with the MBB method (HPLC, panels A-D), potentiometric method (ion-selective electrode, panel E), spectrophotometric method (methylene blue method, panel F), (mean ± SEM). Controls (white bar); patients (black bar). *p < 0.05, **p ≤ 0.01, ***p < 0.001.

Table 2
Values of the various relevant sulfur metabolites (mean ± SEM).

	Controls	Patients
Hcy (μM)	13.50 ± 2.6	35.95 ± 2.5***
Cys (μM)	76.44 ± 2.7	145.3 ± 7.1***
Free Hcy (μM)	0.68 ± 0.08	1.29 ± 0.09***
Cystathionine (μM)	<0.002[a]	5.42 ± 0.5***
Homoserine (μM)	6.13 ± 0.2	5.53 ± 0.3*
Lanthionine (μM)	<0.002[a]	0.32 ± 0.03***
Homolanthionine (μM)	0.26 ± 0.04	0.52 ± 0.1*

*p < 0.05,***p < 0.001.
[a] Detection limit.

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 H_2S 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 H_2S, in both types of dialysis, did not vary significantly (**panel A, B, G, H**).

H_2S measured with potentiometric method did not show any significant variation during HD and PHF dialysis; also H_2S measured with spectrophotometric method did not shown any significant variation during HD and PHF dialysis (not shown).

Table 3
Values of H_2S with the three different methods (mean ± SEM).

H_2S (μM)	HD low-flux polysulfone	HD low-flux polyethersulfone	HDF high-flux polysulfone	PHF high-flux polyethersulfone
Monobromobimane method, HPLC (Free)	8.16 ± 0.2[a***]	4.04 ± 0.4	8.23 ± 0.6	3.40 ± 0.5[b***]
Monobromobimane method, HPLC (boundsulfane sulfur)	2.03 ± 0.2	2.1 ± 0.3	3.02 ± 0.6[c*]	1.36 ± 0.3
Monobromobimane method, HPLC (Acid-labile sulfide)	4.11 ± 0.4	5.57 ± 0.4	4.50 ± 0.9	5.82 ± 0.6
Monobromobimane method, HPLC (Total)	14.06 ± 0.5[a*]	11.85 ± 0.7	16.10 ± 1.5[b*]	10.60 ± 0.9
Ion-selective electrode	75.32 ± 2.1[a**]	58.68 ± 8.6	74.39 ± 2.8	53.87 ± 8.1[b**]
Methylene blue method	26.93 ± 3.8	36.63 ± 4.6	24.53 ± 3.6	38.53 ± 2.5[b*]

*p < 0.05, **p ≤ 0.01, ***p < 0.001.
[a] HD polysulfonevs HD polyethersulfone.
[b] HDF polysulfonevs PHF polyethersulfone.
[c] HD polysulfonevs HDF polysulfone.

Table 4
H_2S measured in its various forms with the MBB method (HPLC), potentiometric method (ion-selective electrode), and spectrophotometric method (methylene blue method) before (PRE) and after dialysis (POST) (mean ± SEM).

H_2S (μM)	PRE	POST
Monobromobimane method, HPLC (Free)	3.83 ± 0.3	3.80 ± 0.3
Monobromobimane method, HPLC (boundsulfane sulfur)	1.49 ± 0.2	1.57 ± 0.2
Monobromobimane method, HPLC (Acid-labile sulfide)	5.74 ± 0.3	4.44 ± 0.3***
Monobromobimane method, HPLC (Total)	11.45 ± 0.6	9.6 ± 0.5**
Ion-selective electrode	73.21 ± 9.9	79.86 ± 10.6**
Methylene blue method	34.16 ± 3.5	29.75 ± 3.9

*p < 0.05,**p ≤ 0.01,***p < 0.001.

Table 5
Relevant sulfur metabolites levels before (PRE) and after dialysis (POST), (mean ± SEM).

	PRE	POST
Hcy (μM)	39.98 ± 6.78	26.48 ± 5.6***
Cys (μM)	139.2 ± 13.73	64.31 ± 6.8***
Free Hcy (μM)	1.59 ± 0.4	1.44 ± 0.3
Cystathionine (μM)	6.83 ± 1.6	1.59 ± 0.5**
Homoserine (μM)	5.45 ± 0.8	4.03 ± 0.3
Lanthionine (μM)	0.34 ± 0.06	0.15 ± 0.03**
Homolanthionine (μM)	0.22 ± 0.03	0.13 ± 0.05

**p ≤ 0.01, ***p < 0.001.

3.4. Lanthionine inhibits H_2S 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 H_2S 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 H_2S production, and the regulatory properties of the major enzymes involved, CBS and CSE. HepG2 cells were incubated with various substrates and cofactors for H_2S 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 H_2S 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 H_2S produced after 24 h of incubation was generally higher than that produced after 6 h (**panel B vs panel A**). H_2S release in cells incubated with Hcy, Cys, *S*-adenosylmethionine (AdoMet) and vitamin B_6 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 H_2S produced by cells during 6 h incubation under conditions of maximal H_2S 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 H_2S 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 H_2S 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 H_2S 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].

H_2S is involved in modulation of various physiological responses. In general, it acts as a protective agent in oxidation, 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 H_2S 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]. Aminzadeh and Vaziri also demonstrated low H_2S levels in a rat model of CKD; the alteration is linked to CKD progression [3].

The best method for H_2S measurement has caused considerable

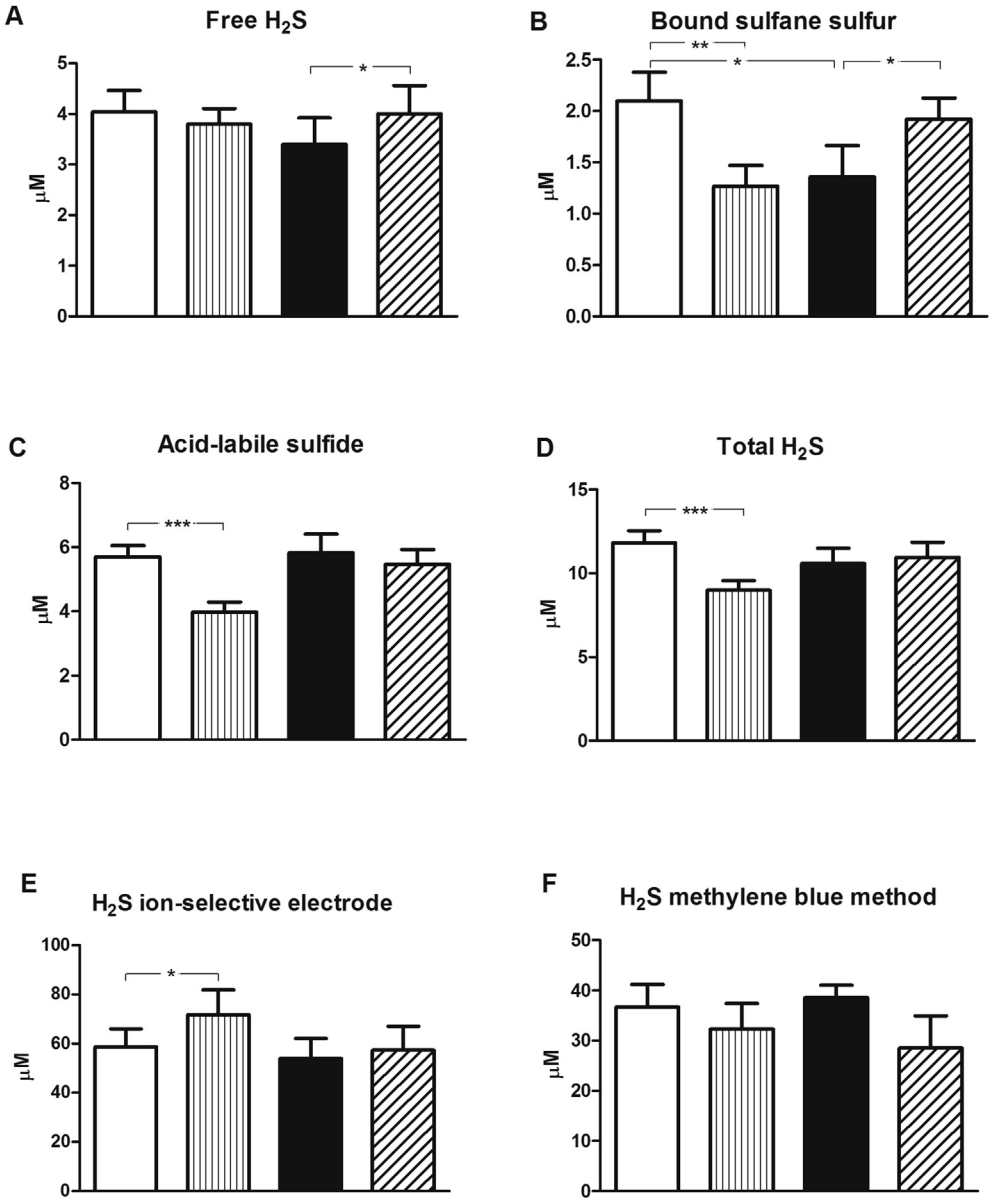

Fig. 4. H_2S measured in its various forms with the MBB method (HPLC), potentiometric method (ion-selective electrode), spectrophotometric method (methylene blue method), before (PRE) and after dialysis (POST) in HD polyethersulfone and PHF (mean ± SEM). PRE HD (white bar), POST HD (vertical line bar), PRE PHF (black bar), POST PHF (cross-hatched bar). $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p < 0.001$.

controversy. H_2S exists in various chemical forms: free (hydrosulphide anion, HS^-), acid-labile (bound to the sulfur-iron clusters of proteins), bound sulfane sulfur (bound to proteins, *i.e.* polysulfides, persulfides, etc.) [10,19]. Total H_2S refers to all these forms together. Total H_2S 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_2S, 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-} 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_2S dispersion. In addition, all H_2S forms can be measured separately. In this article, all three methods were utilized and compared.

In our work, plasma H_2S 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_2S 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_2S generating reactions, *i.e.*Cys β-replacement and Hcy γ-replacement, yield lanthionine or homolanthionine [8], two novel sulfur metabolites, which have been proposed to be useful for monitoring H_2S production.

Our results show that, like all the other sulfur metabolites, lanthionine and homolanthionine are increased in uremic patients

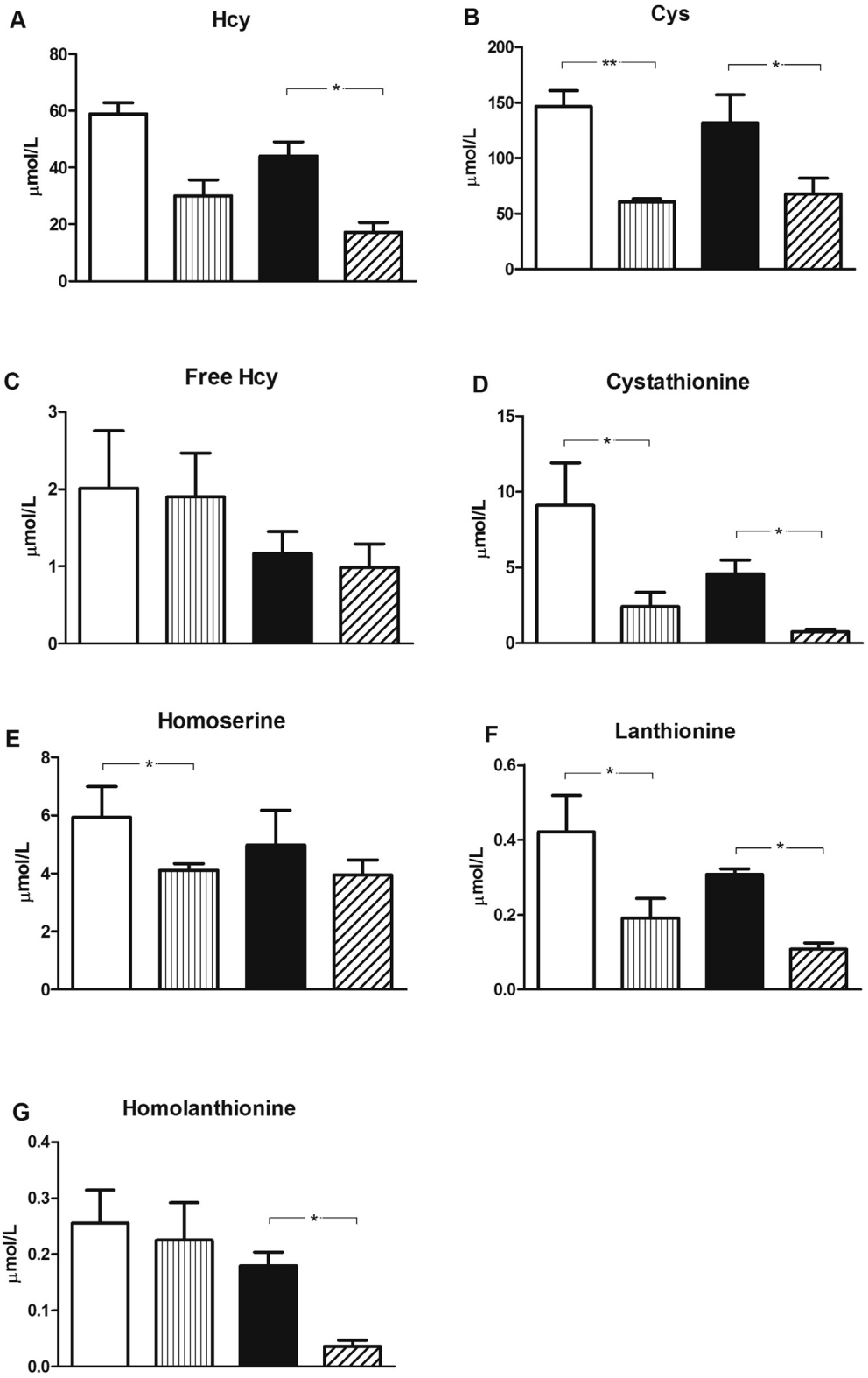

Fig. 5. Concentrations of the various sulfur metabolites, before (PRE) and after dialysis (POST) in HD polyethersulfone and PHF (mean ± SEM). PRE HD (white bar), POST HD (vertical line bar), PRE PHF (black bar), POST PHF (cross-hatched bar). *$p < 0.05$, **$p \leq 0.01$.

with respect to control subjects, despite the above mentioned opposite modifications in H_2S 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 H_2S 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

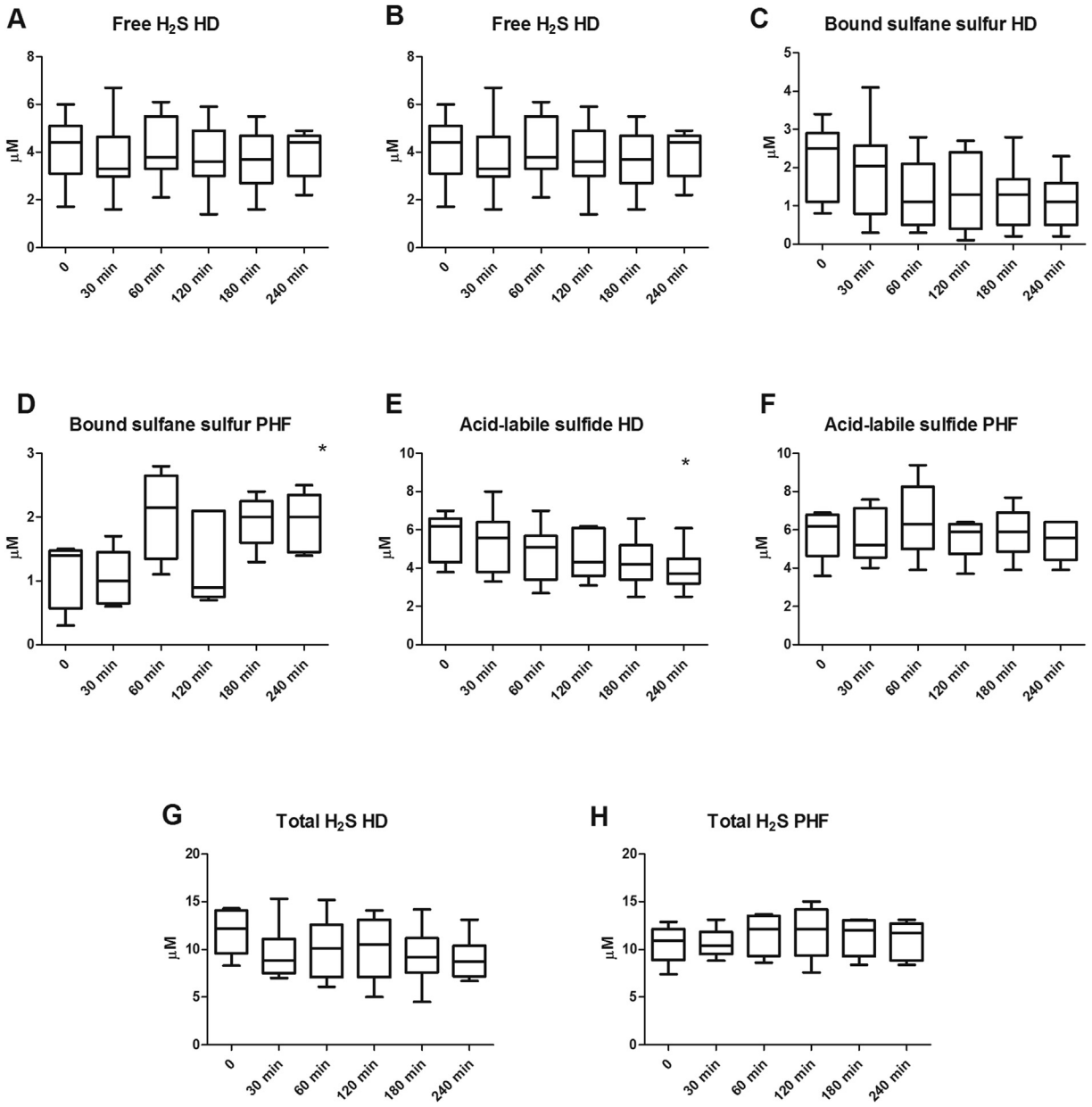

Fig. 6. Time course of the various forms of H_2S, in a single dialysis session, in HD polyethersulfone and PHF (mean & whiskers min to max).*$p < 0.05$ **$p \leq 0.01$.

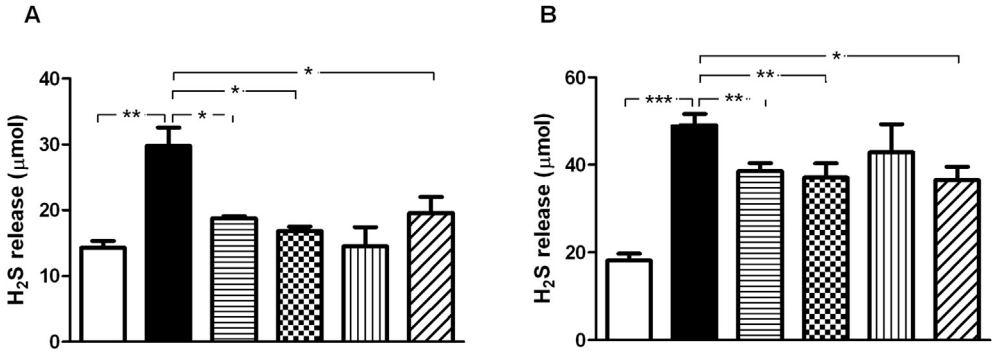

Fig. 7. Amount of H_2S produced by HepG2 cells incubated with various substrates. Panel A) 6 h of incubation; panel B) 24 h of incubation (mean ± SEM). *$p < 0.05$, **$p \leq 0.01$, ***$p < 0.001$. White bar = Control. Black bar = DL-Hcy 10 mM + L-Cys 10 mM + AdoMet 1 µM + vitamin B_6 6 mM. Horizontal 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 B_6 6 mM. Checkered bar = DL-Hcy 10 mM + L-Cys 10 mM + vitamin B_6 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 + vitamin B_6 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 B_6 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 H_2S 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 H_2S production in uremia, compared to homolanthione and lanthionine.

The type of dialysis influences the levels of the different forms of H_2S. Comparing the four different types of dialysis (low-flux HD with polysulfone and polyetersulfone, and high-flux HDF with polysulfone and PHF with polyetersulfone), it emerges that the bound form and accordingly the total H_2S 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 H_2S 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 H_2S [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.

Homolanthionine 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 H_2S 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 H_2S form concentration, and in terms of lanthionine removal.

5. Conclusions

This work underlines the value of measuring H_2S 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 H_2S production, emerges as novel uremic retention product, recommending cautious assessment of parameters of renal function before assuming that lanthionine or homolanthionine levels may adequately monitor H_2S production *in vivo*. Lanthionine accumulation is able to hamper H_2S 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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