



Human ultrarare genetic disorders of sulfur metabolism demonstrate redundancies in H₂S homeostasis

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ABSTRACT

Regulation of H₂S homeostasis in humans is poorly understood. Therefore, we assessed the importance of individual enzymes in synthesis and catabolism of H₂S by studying patients with respective genetic defects. We analyzed sulfur compounds (including bioavailable sulfide) in 37 untreated or insufficiently treated patients with seven ultrarare enzyme deficiencies and compared them to 63 controls. Surprisingly, we observed that patients with severe deficiency in cystathionine β -synthase (CBS) or cystathionine γ -lyase (CSE) - the enzymes primarily responsible for H₂S synthesis - exhibited increased and normal levels of bioavailable sulfide, respectively. However, an approximately 21-fold increase of urinary homolanthionine in CBS deficiency strongly suggests that lacking CBS activity is compensated for by an increase in CSE-dependent H₂S synthesis from accumulating homocysteine, which suggests a control of H₂S homeostasis *in vivo*. In deficiency of sulfide:quinone oxidoreductase - the first enzyme in mitochondrial H₂S oxidation - we found normal H₂S concentrations in a symptomatic patient

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and his asymptomatic sibling, and elevated levels in an asymptomatic sibling, challenging the requirement for this enzyme in catabolizing H₂S under physiological conditions. Patients with ethylmalonic encephalopathy and sulfite oxidase/molybdenum cofactor deficiencies exhibited massive accumulation of thiosulfate and sulfite with formation of large amounts of S-sulfocysteine and S-sulfohomocysteine, increased renal losses of sulfur compounds and concomitant strong reduction in plasma total cysteine. Our results demonstrate the value of a comprehensive assessment of sulfur compounds in severe disorders of homocysteine/cysteine metabolism and provide evidence for redundancy and compensatory mechanisms in the maintenance of H₂S homeostasis.

Abbreviations

AASA	α-aminoadipic semialdehyde	Hcy	homocysteine
AST	L-aspartate:2-oxoglutarate aminotransferase	MoC	molybdenum cofactor
AOX	aldehyde oxidase	MoCD	molybdenum cofactor deficiencies
CARS2	cysteinyl tRNA synthase 2	MoCD-A	molybdenum cofactor deficiency A due to cyclic pyranopterin monophosphate synthase deficiency
CBS	cystathionine β-synthase	MoCD-B	molybdenum deficiency B due to molybdopterin synthase deficiency
CBSD	cystathionine β-synthase deficiency	MPST	mercaptopyruvate sulfurtransferase
CDO	cysteine dioxygenase	MTO	methanethiol oxidase
CSE	cystathionine γ-lyase (also γ-cystathionase, also abbreviated as CGL)	PDO	persulfide dioxygenase
CSED	cystathionine γ-lyase deficiency	PLP	pyridoxal 5'-phosphate
CSAD	cysteine sulfinic acid decarboxylase	SSC	S-sulfocysteine
Cys	cysteine	SSH	S-sulfohomocysteine
EF	excretional fraction	SOX	sulfite oxidase
EE	ethylmalonic encephalopathy due to persulfides dioxygenase deficiency	SOXD	sulfite oxidase deficiency
GSH	glutathione	SQOR	sulfide:quinone oxidoreductase
GSSH	glutathione persulfide	SQORD	sulfide:quinone oxidoreductase deficiency
H ₂ S	hydrogen sulfide	tCys	total cysteine
		tHcy	total homocysteine
		TST	thiosulfate sulfur-transferase

1. Introduction

Hydrogen sulfide (H₂S) has been implicated in the early anoxic phase of life evolution [1] and its role in physiological processes such as vasodilation, neuromodulation and immunomodulation has been reviewed extensively [2,3]. Many studies showed disturbed H₂S homeostasis in common disorders such as cardiovascular disease, insulin resistance and cancer, with modest but statistically significant alterations in H₂S plasma levels [2-4]. In addition, H₂S metabolism has been explored as a novel therapeutic target [5]. However, assessing concentrations of H₂S and related sulfur compounds is not trivial, due to their high reactivity, protein binding, polysulfide formation, dynamic and rapid interconversions and analytical intricacies when using different reagents for their detection [6-8]. The human body receives H₂S via multiple routes, namely from polysulfides and other precursors in food, from intestinal microbiota and via endogenous synthesis routes (Fig. 1). The relative contribution of endogenous and exogenous sources to the body pool of H₂S is at present unknown.

Endogenous H₂S synthesis is intimately linked to cysteine (Cys) metabolism. Derived from the diet or the transsulfuration pathway, Cys that is not used for protein synthesis is primarily incorporated into glutathione (GSH) or it is converted via cysteine sulfinic acid to taurine by the subsequent reactions catalyzed by cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD, Fig. 1). Alternatively, aspartate:2-oxoglutarate aminotransferase (AST) converts cysteine sulfinic acid to β-sulfinyl pyruvate, which spontaneously decomposes to pyruvate and sulfite. The latter is terminally oxidized to sulfate by mitochondrial sulfite oxidase (SOX).

In the past few decades, alternative pathways of Cys metabolism, which produce H₂S, have been described. At least five different enzymes were shown to produce H₂S endogenously using Cys and/or cystine and

to a lesser extent also homocysteine (Hcy; Fig. 1). Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) catalyze — in addition to their canonical reactions — condensation of two sulfur amino acids resulting in H₂S elimination [9]. An alternative route for H₂S synthesis could be the AST/MPST (mercaptopyruvate sulfurtransferase) system transforming Cys via mercaptopyruvate into H₂S and pyruvate. Recently, we have shown that a sulfite-dependent increase in cellular H₂S production requires AST2 (GOT2) expression while CSE and CBS were repressed under conditions of sulfite excess [10]. Methanethiol oxidase (MTO) can also produce H₂S [11]. Another mechanism for H₂S production involves its release from persulfidated species that are either the product of H₂S-dependent reactions (see below) or of a novel reaction of cysteinyl tRNA synthase 2 (CARS2) or of CSE and CBS using cystine as substrate (CysSSCys → CysSSH) [12,13]. Quantitative modeling of fluxes of sulfur metabolites on the basis of the kinetic parameters of involved enzymes and known substrate concentrations suggested a significant contribution of CSE [9,14-16]. However, these models did not (and cannot) take into account variation due to 1) diffusion rates, 2) localization of enzymes in close proximity of transporters, 3) altered general or tissue-specific expression of transsulfuration enzymes under pathological conditions or 4) activation of alternative metabolic pathways due to accumulation of substrates in certain conditions. Therefore, in our view the contributions of different pathways to the production of H₂S and other sulfur species *in vivo* needs to be investigated in an intact biological system and under both physiological and pathological conditions.

Catabolism of H₂S is localized to mitochondria [15,17-20]. In the first step, H₂S is converted to glutathione persulfide (GSSH) by sulfide:quinone oxidoreductase (SQOR). Persulfidated GSH is either converted to sulfite and GSH by persulfide dioxygenase (PDO, also called ETHE1 protein) or alternatively to thiosulfate and GSH by thiosulfate sulfur-transferase (TST). Alternatively, thioredoxin and GSH-dependent enzymes are able to liberate H₂S thus controlling total H₂S levels. TST is

Table 1

Key features of human genetic disorders in sulfur metabolism of enzyme deficiencies.

Enzyme deficiency/disease	Clinical features	Disturbances in sulfur metabolism described	Estimated number of patients known
H₂S synthesizing enzymes			
<u>CBS</u>	Severe to moderate: thromboembolism, marfanoid features, lens dislocation, osteoporosis, cognitive impairment	Strongly increased plasma tHcy, slight increase in H ₂ S production from alternative Hcy metabolism to homolanthionine	>1,000
<u>CSE</u>	Probably benign, originally described in patients with cognitive impairment, but also found with normal development, no reported hypertension	Elevated cystathionine, normal to elevated plasma tHcy	unknown
MPSTD	Probably benign; originally described in patients with cognitive impairment, subsequently not confirmed	Mercaptolactate and mercaptopyruvate elevation in urine	<5 cases
CARS2D	Severe. Progressive myoclonic epilepsy, neurodegeneration.	No data on sulfur compounds	<5 cases
MTOD	Benign: extraoral halitosis	Methanethiol, dimethylsulfide and dimethylsulfoxide accumulation	≈5 cases
H₂S and Cys catabolizing enzymes			
<u>SQORD</u>	Acute Leigh syndrome-like presentation, potentially fatal; may be asymptomatic	No data on sulfur metabolism reported	2 families
<u>EE</u>	Severe: seizures, cognitive impairment, movement disorder, severe diarrhea, vascular petechial purpura and orthostatic acrocyanosis)	Elevated thiosulfate in urine, secondary inhibition of cytochrome c-oxidase by accumulating H ₂ S	>50
CDOD	Not described in humans		
TSTD	Not described in humans		
Defects in oxidation of sulfite to sulfate			
<u>MoCD</u>	Severe: neonatal onset epileptic encephalopathy, neuronal necrosis, brain atrophy, myoclonus, spasticity, cognitive impairment, lens dislocation, xanthine urolithiasis	Decreased tHcy, elevated sulfite, thiosulfate and S-sulfocysteine in plasma and urine	>200 cases
<u>SOXD</u>	Severe: neonatal onset epileptic encephalopathy, neuronal necrosis, brain atrophy, myoclonus, spasticity, cognitive impairment, lens dislocation,	Decreased tHcy, elevated sulfite, thiosulfate and S-sulfocysteine in plasma and urine	>100 cases

Bold underlined enzyme deficiencies addressed in this study; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; MPSTD, mercaptopyruvate sulfurtransferase; CARS2, cysteinyl tRNA synthase 2; MTO, methanethiol oxidase; SQORD, sulfide:quionone oxidoreductase; EE, ethylmalonic encephalopathy due to persulfide dioxygenase deficiency; CDO, cysteine dioxygenase; TST, thiosulfate transferase; MoC, molybdenum cofactor; SOX, sulfite oxidase; tHcy, total homocysteine. For details on clinical and biochemical features see Refs. [36–38].

2. Methods

2.1. Patients

Information about this study was disseminated via the European Reference Network for Hereditary Metabolic Disorders and presentation at conferences. Interested physicians received information for patients and informed consent forms in Czech or English. Clinical, enzymatic, genetic and therapy details from consenting patients were obtained and diagnosis was verified by inspecting the data (data not shown and will be published in a separate study). For the present study, we used only samples from patients in a metabolic steady-state, obtained prior to the start of specific therapy or during episodes of non-compliance (in CBS deficient patients) or from patients undergoing treatments not likely to substantially alter their metabolic state. The study cohort consisted of individuals with pyridoxine non-responsive CBS deficiency (CBS), total n = 14; untreated patients, n = 3; patients with total Hcy >100 μmol/L, i.e. higher than the generally accepted target range indicating sufficient biochemical control [39], n = 11), pyridoxine non-responsive CSE deficiency (CSED; n = 1), SQORD deficiency (SQORD; n = 3; family B reported in Ref. [40]; two independent samples obtained from the index case and single samples from two asymptomatic sisters), ethylmalonic encephalopathy (EE; n = 7), MoC deficiency A (MoCD-A) and MoC deficiency B (MoCD-B; n = 2 and n = 7, respectively), and SOX deficiency (SOXD; n = 3).

Control samples from healthy individuals on a normal western diet were collected prospectively in a previous study [41] (n = 12) and in the present study (n = 51), the final group of 63 controls comprised 17 males and 38 females with a median age of 37 years (range 6–58 years), and 8 anonymous controls with unknown sex and age.

2.2. Sample collection and processing

After a standard venepuncture blood was collected into lithium-heparin vacutainer tubes with gel (BD Vacutainer LH PST II) at least 2–3 h after the last meal, and immediately placed into an ice/water slush, plasma was separated by centrifugation (2,000 g at 4°C for 5 min) within 10–30 min after collection. Freshly voided urine samples were immediately cooled in the ice/water slush. Urine and separated plasma were immediately frozen at –85°C and stored at –85°C for up to 3 months prior to analysis (except for samples from patients MoCD-A-01, MoCD-B-02, MoCD-B-03 and SOXD-02 that were stored for more than 1 year), transport of samples between clinical centres and laboratories used dry ice as a cooling medium. To estimate the stability of analytes at –85°C we utilized retrospective and prospective data from serial analyses of control materials. Pooled control plasma and urine samples were aliquoted, stored at –85°C between 133 and 260 days and measured repeatedly in each series of patient samples, regression model was used to estimate changes after 90 days. All analytes were stable with predicted changes <10% of initial concentrations, except of less stable S-sulfocysteine and thiosulfate in plasma with predicted decrease by 37% and 32.2%, respectively. Details are shown in Supplementary Method SM5.

2.3. Ethics

Samples from patients and controls were collected between October 2017 and June 2020, informed consent from participants was obtained. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the General University Hospital in Prague (approval Nr. Grant COST 35/13, Nr.AZV 71/15 and Nr.2130/18 IS).

2.4. Determination of sulfur containing compounds, alpha-aminoadipic semialdehyde (AASA) and vitamin B₆ vitamers

Metabolites were determined by methods described in detail elsewhere [6,41] or in the Supplementary Methods SM1-SM4. Reverse phase HPLC separation of fluorescently labelled compounds was used for

analysis of bioavailable sulfide, sulfite, thiosulfate, and of total Cys (tCys) and total Hcy (tHcy). LC-MS/MS was used to determine cystathionine, homolanthionine, lanthionine, taurine, S-sulfocysteine (SSC) and S-sulfohomocysteine (SSH), α -aminoadipic semialdehyde (AASA) and vitamin B₆ vitamers. Capillary electrophoresis was used to analyse sulfate.

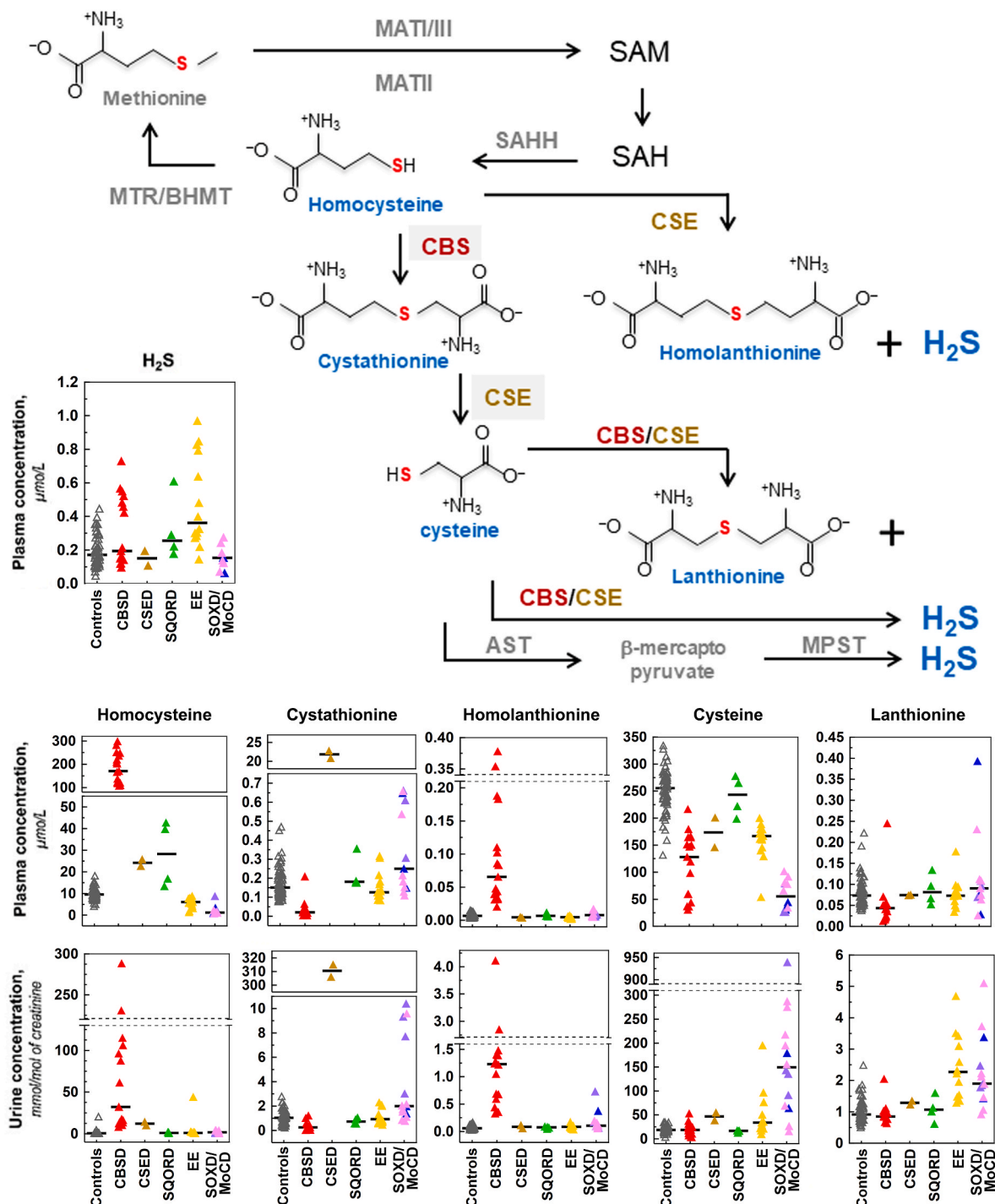


Fig. 2. Concentrations of metabolites relating to H₂S synthesis. Reactions leading to the formation of H₂S metabolites are shown in the proximal part of the sulfur metabolic pathway; blue labelled metabolites were determined in this study. The color codes of enzymes depicted in this figure and Fig. 3 are matched with the color code of patient groups (grey, controls; red, CBS/CBSD; brown, CSE/CSED; green, SQOR/SQORD; yellow, EE; blue, SOX/SOXD; magenta, MoCD). SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MATI/III, methionine adenosyltransferase I/III; MATII, methionine adenosyltransferase II; MTR, methionine synthase; BHMT, betaine:homocysteine methyltransferase; SAHH, S-adenosylhomocysteine hydrolase, CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; AST, aspartate aminotransferase; MPST, mercaptopyruvate sulfurtransferase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Assessment of renal handling of metabolites

The fractional excretion (excretional fraction, EF) of metabolites was calculated using the standard formula: $EF_{\text{metabolite}} [\%] = 100 \times (U_{\text{metabolite}} \times P_{\text{creatinine}}) / (P_{\text{metabolite}} \times U_{\text{creatinine}})$, where U and P are concentrations of analytes determined in simultaneously obtained urine and plasma samples, respectively. Creatinine in urine and plasma were determined by local clinical biochemistry laboratories.

2.6. Statistical analyses

Samples were processed in batches in a blinded fashion, each series of analyses contained both control and patient samples, and blanks. The number of plasma and/or urine samples varied between 1 and 5 per

patient. To describe typical concentrations of metabolites in each disease cohort, we first calculated median concentrations of analytes for each individual if multiple samples were available, and then established the median for each cohort of patients with the same disease. Data for disease cohorts are shown as medians with ranges. In controls we calculated medians and the 2.5th and 97.5th percentile. Due to the small numbers of patients in each disease category, we were unable to perform formal statistical testing.

3. Results and discussion

3.1. Concentration of sulfur metabolites in plasma and urine

In this study we explored the role of selected enzymes in maintaining

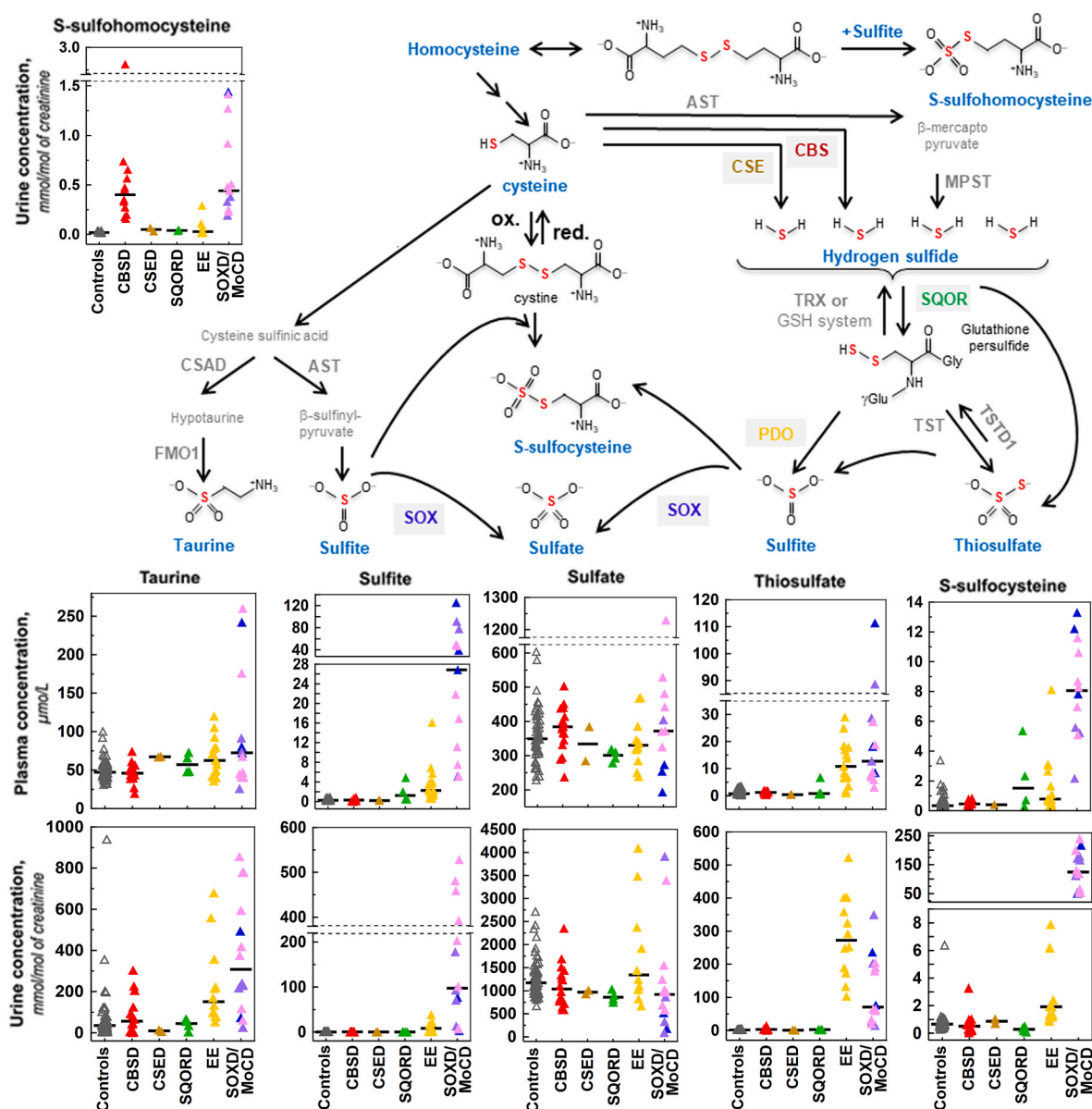


Fig. 3. Concentrations of metabolites relating to H₂S catabolism. Reactions in catabolism of H₂S and sulfite are shown in the distal part of the sulfur metabolic pathway; blue labelled metabolites were determined in this study. The color codes of enzymes depicted in this figure and Fig. 2 are matched with the color code of patient groups (grey, controls; red, CBS/CBSD; brown, CSE/CSED; yellow, EE; green, SQOR/SQORD; blue, SOX/SOXD; magenta, MoCD). CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; AST, aspartate aminotransferase; CSAD, cysteinylsulfinate decarboxylase; CDO, cysteine dioxygenase; TRX, thioredoxine; FMO1, flavin containing dimethylalanine monooxygenase 1; MPST, mercaptopyruvate sulfurtransferase; SQOR, sulfide:quinone oxidoreductase; SOX, sulfite oxidase; PDO, persulfide dioxygenase; TST, thiosulfate transferase; TSTD1, thiosulfate:glutathione sulfurtransferase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

homeostasis of H₂S, sulfite and related sulfur compounds by analyzing sulfur-containing metabolites in plasma and urine samples from three groups of patients with ultra-rare genetic disorders, namely with deficient activity of two H₂S synthesizing enzymes, two enzymes with defects in conversion of H₂S to sulfite, and three groups of patients with a block in the oxidation of sulfite to sulfate. Remarkably, for some metabolites, concentration changes showed an extreme range over several orders of magnitude, whereas plasma concentrations of bioavailable sulfide and other metabolites remained within a much narrower range (Figs. 2–4 and Supplementary Tables 1–3). The typical concentrations of metabolites in controls and each disease cohort are shown as medians and ranges in Table 2. To illustrate the large effect size, we provide also graphical, logarithmical representation of results in form of radial charts (Supplementary Fig. 1).

3.1.1. Substrates and byproducts of enzymatic synthesis of H₂S

Cys is the major substrate for H₂S synthesis by at least four enzymes (Fig. 1), while CSE -but not CBS- can also catalyze H₂S synthesis using two Hcy molecules as a substrate with the concomitant production of homolanthionine [14,42,43]. CBS and CSE exhibit relaxed substrate specificity towards hydroxyl- and thiol-containing amino acids (Fig. 2) and may release water or H₂S with a simultaneous synthesis of the thioethers cystathionine, homolanthionine and lanthionine [14,42]. The two latter thioethers may serve as indirect markers of H₂S synthesis [43].

Plasma total homocysteine is a mixture of small amounts of reduced Hcy, its disulfides and a larger proportion of protein bound Hcy [44,45]. Measuring individual Hcy fractions requires specific pre-analytical conditions that are difficult to meet in clinical settings [46,47]. tHcy correlates well with its plasma fractions in a range up to approximately 150 μmol/l [48] and is generally accepted as a robust proxy marker of extracellular homocysteine accumulation. We have discussed the choice of tHcy as biomarker further in Supplementary Material SR1.

Median plasma concentrations of plasma tHcy were elevated in samples from CBSD (≈18-fold), CSED (≈2.5-fold) and SQORD patients (≈1.8-fold). In contrast, plasma samples from EE patients were borderline low (median ≈70% of controls) and very low in SOXD/MoCD patients (median ≈15% of controls). Urine concentrations of tHcy were increased ≈77-fold, ≈23-fold and ≈3.5-fold in CBSD, CSED and SOXD/MoCD patients, respectively. Plasma and urinary tHcy levels were within the reference range in patients with other diseases.

Cystathionine originates mostly from condensation of serine and Hcy by CBS [49] or potentially from synthesis from a non-canonical CSE reaction, while its removal is catalyzed by CSE. Congruently, plasma cystathionine concentrations were decreased to ≈15% of normal in CBSD patients [37,38] and increased ≈135-times in the CSED patient.

Urinary concentration was extremely elevated in the CSED (≈290-times) patient while in the CBSD patients the urinary concentration was decreased to ≈30% of controls but overlapped with the reference range. Cystathionine was elevated in plasma and especially in urine from some SOXD/MoCD patients, however, the median of this group was still within the high reference range.

Homolanthionine is considered a marker of CSE-catalyzed synthesis of H₂S from Hcy [16,41]. It was consequently elevated ≈11-fold and ≈21-fold in plasma and urine, respectively, that was obtained from CBSD patients with severely increased tHcy, and surprisingly in a few urine samples from SOXD/MoCD patients leading to ≈2.3-fold increase of the median.

Total cysteine is the sum of reduced, oxidized and protein bound Cys fractions, which exist in a dynamic equilibrium together with other aminothiols (see also Supplementary Results SR1). The reducing agent tris(2-carboxyethyl) phosphine used in our method releases Cys also from SSC (>90% of S-sulfocysteine is converted to tCys after incubation with the reducing agent, data not shown) and the method therefore overestimates tCys concentrations when SSC is markedly elevated (as seen in SOXD and MoCD). Plasma tCys was within the reference range in SQORD patients, but clearly decreased to ≈50%, ≈70%, ≈60% and ≈20% of normal in patients with CBSD, CSED, EE and SOXD/MoCD, respectively.

Cysteine and cystine are provided from dietary protein and will therefore be present in plasma of patients with CBSD despite a block in conversion of Hcy to Cys, albeit in decreased amounts. The strongly elevated Hcy concentration in untreated CBSD favours the formation of the Hcy-Cys disulfide, where Cys also partly derives from displacement of protein-bound Cys [50]. While this phenomenon will not alter the concentration of tCys in plasma, a significant proportion of tCys will prevail as mixed disulfide which is excreted and poorly re-absorbed in kidneys and will therefore constitute a major proportion of urinary tCys. This explains the relative preservation of tCys concentrations in urine in CBSD despite apparent Cys depletion in plasma [51].

Urinary tCys was increased ~2.5-fold in the CSED patient. This could result from the presence of the mixed Hcy-Cys disulfide in urine, in analogy to the situation in CBSD, but may also result from an inhibition of cystine reabsorption by the very large amount of cystathionine in urine, on the background of the poor specificity of cystine transporters [52]. Cystathionine was recently shown to be a substrate for at least one of the cystine transporters [53]. However, our observation needs to be confirmed in other patients. About 8.5-fold elevated tCys concentration in urine from SOXD/MoCD patients were equal to SSC concentrations and thus simply reflect the high urinary concentrations of SSC in these diseases. Urinary tCys was variably moderately elevated in patients with EE (≈2-fold), correlating with an increased excretion of tHcy and SSC but not fully explained by their presence.

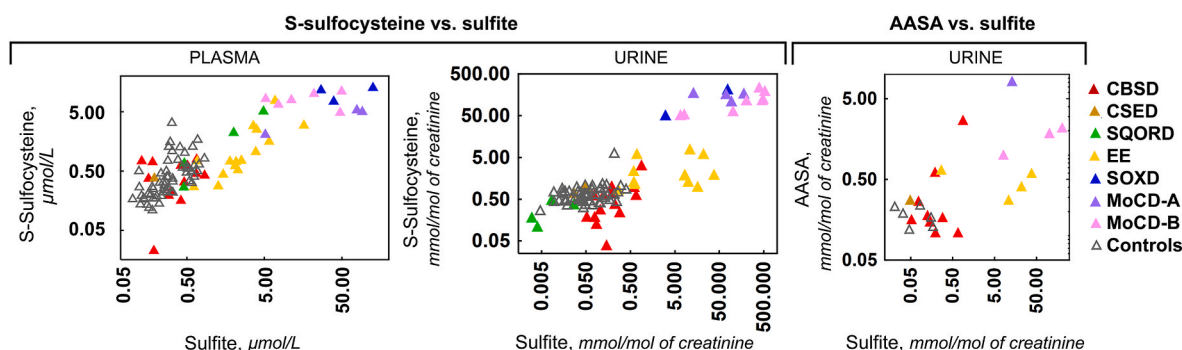


Fig. 4. Correlation between sulfite concentrations and concentrations of S-sulfocysteine and α -aminoadipic semialdehyde. Panel A, correlation between plasma sulfite and plasma S-sulfocysteine (SSC); panel B, correlation between urinary sulfite and urinary SSC; panel C, correlation between urinary sulfite and urinary α -aminoadipic semialdehyde (AASA). Please, note the logarithmic scales of analyte concentrations. Color code of patient groups—red, CBSD; brown, CSED; green, SQORD; yellow, EE; blue, SOXD; magenta, MoCD; grey, controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Concentrations of metabolites in plasma ($\mu\text{mol/L}$), in urine ($\text{mmol/mol of creatinine}$) and excretion fraction EF (%) in patient groups.

		Controls		CBSD		CSED		SQORD		EE		SOXD/MoCD	
		N	Median (2.5th-97.5th centile)	N	Median (Range)	N	Value	N	Median (Range)	N	Median (Range)	N	Median (Range)
H₂S	Plasma	59	0.173 (0.086–0.378)	14	0.263 (0.096–0.566)	1	0.15	3	0.256 (0.177–0.609)	7	0.481 (0.218–0.909)	8	0.146 (0.092–0.258)
S-sulfocysteine	Plasma	56	0.342 (0.131–1.700)	12	0.434 (0.024–0.833)	1	0.40	3	1.31 (0.71–5.35)	7	0.77 (0.29–5.38)	10	8.06 (2.17–13.3)
Total cysteine	Plasma	60	256 (190–321)	14	137 (36.3–217)	1	173	3	222 (199–271)	7	167 (146–200)	10	48.6 (26.2–91.5)
Lanthionine	Plasma	62	0.075 (0.042–0.164)	14	0.044 (0.020–0.245)	1	0.075	3	0.074 (0.067–0.134)	7	0.079 (0.044–0.125)	11	0.091 (0.026–0.393)
Total homocysteine	Plasma	60	9.53 (6.47–15.1)	14	169 (107–298)	1	24.2	3	16.8 (13.4–41.2)	7	6.35 (3.16–7.43)	10	1.27 (0.75–4.96)
Homolanthionine	Plasma	62	0.007 (0.003–0.014)	14	0.075 (0.020–0.378)	1	0.004	3	0.007 (0.006–0.009)	7	0.005 (0.002–0.007)	11	0.008 (0.005–0.016)
Cystathionine	Plasma	62	0.162 (0.077–0.390)	14	0.021 (0.004–0.209)	1	21.8	3	0.185 (0.176–0.266)	7	0.135 (0.086–0.307)	11	0.250 (0.109–0.658)
Sulfite	Plasma	60	0.28 (0.12–0.70)	14	0.33 (0.10–0.67)	1	0.20	3	1.23 (0.46–4.85)	7	2.15 (1.27–9.85)	11	26.9 (5.13–125)
Thiosulfate	Plasma	60	0.86 (0.27–3.24)	14	1.01 (0.31–1.96)	1	0.42	3	0.79 (0.58–6.66)	7	15.07 (1.02–24.88)	11	12.7 (4.51–111)
Sulfate	Plasma	52	347 (243–495)	14	385 (237–503)	1	334	3	294 (292–318)	7	335 (261–468)	10	371 (193–1229)
Taurine	Plasma	61	48.3 (32.7–80.8)	14	46.8 (19.4–74.0)	1	67.2	3	56.7 (48.1–72.8)	7	64.9 (41.5–105)	11	72.4 (40.2–260)
S-sulfocysteine	Urine	54	0.661 (0.333–1.179)	12	0.51 (0.04–3.26)	1	0.87	3	0.28 (0.11–0.49)	6	3.52 (1.18–6.18)	9	129 (51.7–217)
S-sulfohomocysteine	Urine	15	0.020 (0.014–0.037)	10	0.40 (0.16–2.81)	1	0.049	1	0.040	5	0.078 (0.014–0.291)	8	0.46 (0.19–1.44)
Total cysteine	Urine	58	18.4 (9.2–33.9)	14	18.7 (3.74–52.9)	1	46.6	3	16.2 (14.8–17.2)	6	38.1 (21.3–136)	9	156 (21.3–515)
Lanthionine	Urine	58	0.92 (0.64–1.81)	14	0.85 (0.64–2.05)	1	1.29	3	1.01 (0.87–1.60)	6	2.08 (1.35–3.31)	9	2.13 (0.99–5.10)
Total homocysteine	Urine	58	0.53 (0.33–3.12)	14	41.1 (8.2–288)	1	12.1	3	0.86 (0.73–1.70)	6	1.17 (0.58–2.66)	9	1.99 (0.53–4.00)
Homolanthionine	Urine	58	0.059 (0.031–0.131)	14	1.23 (0.33–4.11)	1	0.086	3	0.082 (0.058–0.090)	6	0.096 (0.059–0.115)	9	0.139 (0.080–0.398)
Cystathionine	Urine	58	1.07 (0.30–2.30)	14	0.29 (0.02–1.21)	1	311	3	0.90 (0.56–1.04)	6	1.34 (0.62–2.29)	9	2.03 (0.81–9.59)
Sulfite	Urine	58	0.051 (0.009–0.281)	14	0.164 (0.052–1.13)	1	0.038	3	0.008 (0.004–0.015)	6	6.91 (0.62–24.0)	9	102 (3.07–481)
Thiosulfate	Urine	58	1.50 (0.64–4.14)	14	3.07 (0.50–14.3)	1	0.75	3	2.38 (0.94–2.50)	6	272 (188–522)	9	179 (20.3–276)
Sulfate	Urine	58	1170 (815–2386)	14	934 (587–2350)	1	970	3	900 (785–1030)	6	1546 (670–4090)	9	970 (180–3390)
Taurine	Urine	58	32.4 (2.39–290)	14	56.6 (0.59–303)	1	9.46	3	50.7 (3.91–55.1)	6	207 (52.3–679)	9	374 (73.4–780)
S-sulfocysteine	EF	52	14.0 (3.5–33.6)	11	5.41 (0.19–48.7)	1	8.5	3	2.7 (0.1–3.1)	6	5.4 (3.0–59.7)	6	34.5 (16.8–48.0)
Total cysteine	EF	57	0.5 (0.3–1.0)	13	1.10 (0.50–264)	1	0.9	3	0.3 (0.3–0.4)	6	0.63 (0.30–1.94)	6	6.0 (1.8–10.9)
Lanthionine	EF	57	90.2 (59.3–160)	13	84.9 (0.07–195)	1	54.4	3	68.1 (52.3–74.4)	6	75.1 (23.4–105)	6	82.0 (23.2–185)
Total homocysteine	EF	57	0.4 (0.2–1.9)	13	2.00 (0.40–11.4)	1	1.4	3	0.25 (0.22–0.25)	6	0.82 (0.26–1.51)	6	5.48 (2.97–7.74)
Homolanthionine	EF	57	62.7 (39.0–93.5)	13	68.1 (0.01–85.4)	1	101.1	3	54.4 (44.6–63.8)	6	46.5 (33.6–67.3)	6	49.7 (43.0–97.2)
Cystathionine	EF	57	39.4 (15.5–72.2)	13	38.0 (0.19–189)	1	45.2	3	21.3 (14.6–26.6)	6	19.6 (12.5–30.2)	6	33.0 (14.9–43.8)
Sulfite	EF	57	1.4 (0.1–8.1)	13	1.91 (0.13–54.7)	1	0.8	3	0.061 (0.004–0.082)	6	5.2 (0.6–43.1)	6	14.0 (0.2–44.9)
Thiosulfate	EF	57	9.7 (1.9–50.1)	13	15.3 (0.3–52.2)	1	3.7	3	19.3 (0.6–22.2)	6	96.8 (48.7–527)	6	39.1 (15.9–81.9)
Sulfate	EF	56	24.3 (14.4–41.3)	12	16.4 (5.9–271)	1	10.7	3	15.9 (12.4–16.9)	6	14.1 (5.8–22.2)	6	10.1 (1.8–20.1)
Taurine	EF	57	4.4 (0.4–13.6)	13	6.93 (0.16–56.6)	1	0.7	3	5.2 (0.2–5.4)	6	10.6 (3.6–24.7)	6	15.5 (2.3–25.2)

N, number of subjects in each group; typical concentrations of metabolites and EFs are medians for all patients from the same disease cohort.

Lanthionine produced by both CBS and CSE from either Cys + Cys or serine + Cys was within the reference range in the majority of plasma samples from patients with different disorders (medians of all disorders were within the range of controls). Urinary concentrations were elevated in about half of the samples in EE and SOXD/MoCD patients leading to ≈ 2.3 -fold increase of median in both disorders.

3.1.2. Plasma concentrations of bioavailable sulfide

A newly developed and strictly controlled method [6] that uses a short incubation with monobromobimane determines a pool of readily bioavailable H_2S in plasma, however, H_2S release from persulfidated species cannot be excluded due to the delicate equilibria that exist between these sulfur species in biological systems [7,8]. In contrast to an expected decrease in H_2S levels, we found plasma concentrations being elevated above the reference range in 7 out of 17 CBSD samples (median increase ≈ 1.5 -times) and within the reference range in both CSED samples. Elevation of bioavailable sulfide was observed also in one sample obtained from the asymptomatic SQORD patient and in the majority of EE patients resulting in a median ≈ 2.8 -fold increase in EE patients compared to controls. The alterations in bioavailable sulfide in our study were much stronger than the ones observed in previous studies of human multifactorial diseases, in which less specific methods for H_2S determination were used [54,55].

3.1.3. Catabolism of H_2S and cysteine

Cysteine is metabolized via two different pathways (Fig. 3) yielding either the end-product taurine or a common intermediate of both H_2S and Cys catabolism-sulfite- that can be a/interconverted to thiosulfate by TST, or b/metabolized to the final oxidation product sulfate.

Sulfite concentrations in plasma were increased ≈ 100 -fold in SOXD/MoCD patients, ≈ 8 -fold and ≈ 4.5 -fold in EE and SQORD samples, respectively, while they were within the reference range in CBSD and CSED patients. Urine sulfite concentration was increased ≈ 2000 -fold in patients SOXD/MoCD and ≈ 140 -fold in EE patients, respectively. Slightly increased urinary concentration was also observed in CBSD patients (≈ 3 -fold). In contrast to elevation in plasma, urine from the SQORD patients exhibited low sulfite concentration ($\approx 20\%$ of median of controls).

Plasma **thiosulfate** concentrations were elevated ≈ 18 -times and ≈ 15 -times in EE and SOXD/MoCD patients, respectively, and in urine (≈ 180 -times and ≈ 120 -times, respectively). In CBSD, CSED and SQORD patients, plasma and urine thiosulfate concentrations were usually within the reference range.

Plasma and urine **sulfate** originating from nutrition [56] and from endogenous oxidation of sulfite was usually within the reference range in all patient categories.

Median of plasma **taurine** was usually within the reference range in all patient groups. Urine concentrations were widely dispersed in patients and overlapped with the large reference range in controls. However, the median urine taurine concentration was ≈ 6 -times and ≈ 12 -times higher in the EE and SOXD/MoCD patients than in controls.

3.1.4. Metabolic consequences of sulfite accumulation

Due to its high reactivity, sulfite forms the adducts SSC [57] and SSH non-enzymatically. Moreover, sulfite inhibits the α -aminoacidic semi-aldehyde dehydrogenase leading to accumulation of AASA and its isomer $\Delta 1$ -piperidine-6-carboxylate, which is known to chemically inactivate pyridoxal 5'-phosphate (PLP) [58,59]. However, the relationship between intermediates in sulfur metabolism and vitamin B_6 metabolism is even more complex. Sulfite can react with PLP forming a sulfonate and cysteine can react with PLP to form a thiazolidine. The extent to which the latter occurs *in vivo* and what happens to the thiazolidine that is formed, is unknown.

S-sulfocysteine was increased ≈ 24 -fold and ≈ 195 -fold in SOXD/MoCD plasma and urine, respectively. An increased urinary concentration was also observed in EE patients (≈ 5 -times elevated compared to

the median of controls). SSC was also elevated in half of all plasma samples from patients with SQORD while decreased concentrations in urine were observed in CBSD and SQORD urine samples (to $\approx 80\%$ and $\approx 40\%$ of normal, respectively). SSC in plasma and urine samples from the CSED patient was within the reference range. We observed a direct correlation between sulfite and SSC in both plasma and urine (Fig. 4). Such a correlation has been shown before *in vitro* [57] but not to this extent *in vivo*.

S-sulfohomocysteine concentration was at the detection limit in control plasma samples and we are thus reporting only urinary data. We hypothesized that SSH may be formed non-enzymatically when either Hcy or sulfite accumulate in large quantities. The urine concentration was indeed ≈ 20 -23-times increased in samples from CBSD and SOXD/MoCD patients, respectively, which supports our proposal. Slightly increased SSH concentrations were observed also in some samples obtained from CSED, SQORD and EE patients.

AASA and B_6 vitamers were determined in a small subset of controls and patients from all disease groups except for SQORD (see Supplementary Table 4). Compared to the median of the small group of controls, the median AASA in SOX/MoCD patients was ≈ 17 -times higher (range ≈ 5 -times to 46-times) and well above the appropriate reference range in half of subjects. Although there was a trend towards higher concentrations in EE patients (median ≈ 2.8 -times higher, range ≈ 1.5 to ≈ 3.7), the values were within the reference range (upper limit 2–4 mmol/mol creatinine in different age categories). The association between sulfite and AASA concentrations appears to be linear (Fig. 4, N.B. log-log scale). Plasma PLP concentrations were below that of control samples (range 9.0–21.9 nmol/L) measured at the same time, and that of published ranges [60] for one EE and SOXD patient, respectively. Of further note is that in three of the MoCD patients we also observed a marked increase in the ratio of the B_6 vitamer pyridoxal to its catabolic product pyridoxic acid, which has been reported previously [60]. When B_6 vitamer intake exceeds requirements, pyridoxal phosphate is dephosphorylated (mainly in the liver) and the pyridoxal is oxidized to pyridoxic acid prior to excretion in urine. Early reports suggested that in humans an aldehyde dehydrogenase or aldehyde oxidase (AOX) is responsible for this reaction. However, while in *Drosophila* pyridoxal has been shown to be a substrate of AOX it is not metabolized by mouse AOXs [61]. The increased ratio observed in studied patients would suggest that in humans AOX is involved in the conversion of pyridoxal to pyridoxic acid, as AOX requires MoC for its activity.

3.2. Patterns of metabolic changes in deficiencies of H_2S synthesizing enzymes

3.2.1. CBS deficiency

We observed the expected consequences [36–38] of the impaired canonical CBS reaction such as massive accumulation of the substrate Hcy in body fluids, and decreased plasma concentration of the products cystathionine and Cys in the transsulfuration pathway. Decreased plasma tCys concentrations are expected because of the displacement of Cys from cysteine residues in plasma proteins by Hcy [48] as well as due to decreased production of Cys from lower synthesis of cystathionine. However, Cys is also provided from dietary protein and amino acid mixtures in treated patients, and these nutritional sources partially compensate for its decreased synthesis. The normal tCys excretion in urine in CBSD very likely constitutes an artifact, caused by increased renal clearance of the mixed Cys-Hcy disulfide, as explained above.

Markedly increased SSH in urine support the hypothesis that massive accumulation of Hcy results in formation of SSH. This novel observation requires confirmation by independent studies. It is currently unknown whether accumulating SSH does contribute to a disturbance in neurotransmission in CBSD, similar to the role of SSC in sulfite intoxication disorders [57]. However, it is important to note, that accumulating SSH concentrations were one order of magnitude lower than that of SSC.

The concentration of bioavailable sulfide in plasma varied among

patients and in repeated samples from individual patients if available. Sulfide was elevated above the upper limit of the reference range in almost one half of plasma samples in the cohort of 14 CBSD patients. Elevated urinary excretion of homolanthionine was reported in the 1970s although its origin was attributed to synthesis from Hcy and homoserine by CSE [50]. The extent of homolanthionine accumulation in body fluids in the present study (≈ 11 -fold and ≈ 21 -fold in plasma and urine, respectively) is similar to a previous report [43] that showed ≈ 32 -fold increase in plasma of 14 untreated CBS deficient patients. Taken together, all these observations are congruent with a model whereby in CBSD the massively elevated homocysteine becomes the dominant H₂S donor via an alternative CSE reaction [16].

3.2.2. CSE deficiency

Despite significant international networking efforts, we only succeeded in collecting two plasma and urine samples from one pyridoxine non-responsive patient with profound deficiency of CSE, who was diagnosed during a workup for behavioral problems. The expected findings [62] included increased accumulation of tHcy and cystathionine in body fluids. Although CSE plays an important role in the transsulfuration pathway, we have not observed lower sulfate in plasma or urine; moderately decreased tCys in plasma is probably caused by a mechanism similar to CBSD (complex equilibrium of reduced, oxidized and protein bound aminothiols). In contrast to reports on decreased H₂S levels in CSE-deficient mice [31], plasma bioavailable sulfide was within the reference range in two plasma samples obtained from this patient during different visits. Discrepant results of our study in a human patient and in the study of CSE-deficient mice originate most likely from the use of different methods. The monobromobimane method used in our study determined nanomolar concentrations of bioavailable sulfide (i.e., free sulfide and a fraction of easily liberated sulfide). In contrast, the study in CSE-deficient mice used sulfide selective electrode with the Sulfide AntiOxidant Buffer that also liberates H₂S from its oxidized forms, which is also reflected as very high blood H₂S concentrations reaching dozens of micromoles per liter (i.e. concentrations considered highly toxic). Another source of variation that cannot be excluded may be interspecies differences in regulatory networks of H₂S homeostasis. Homolanthionine is present in nanomolar concentrations in controls and levels in the CSED patient were within the reference range in all analyzed samples. It is unlikely that the patient may have any residual CSE activity capable of producing H₂S as he carries a large homozygous deletion in the *CTH* gene spanning four exons. This raises the question about the origin of homolanthionine in a patient with completely inactive CSE.

3.2.3. Complementary and partial functions of CBS and CSE in H₂S synthesis

According to the current paradigms in the field [16], decreased H₂S synthesis and possibly low free sulfide levels would have been expected in patients with CBS and CSE deficiencies. However, we observed an increase in CBSD patients and a normal concentration of H₂S in the CSED patient. We also found a massive accumulation of plasma and urine homolanthionine in CBS deficiency, which suggests that the lack of CBS-dependent H₂S synthesis is compensated for by Hcy-dependent H₂S synthesis via CSE. These observations raise an important question on the relative role of nutritional, microbial and enzymatic origin of H₂S in the human body and lend support to a hypothesis that deficiency of a single H₂S producing enzyme in the complex whole body homeostatic network regulating H₂S synthesis is compensated for by other sources.

3.3. Patterns of metabolic changes in deficiencies of enzymes converting H₂S to sulfite

3.3.1. SQOR deficiency

Sulfur metabolism in patients with deficient SQOR activity has not yet been reported although accumulation of H₂S was implied as the cause of decreased cytochrome c-oxidase activity in tissues from a

deceased patient [40]. We hypothesized that the SQORD patients will have increased free sulfide and decreased GSSH and Cys persulfide resulting in a possibly decreased sulfite production [63]. In this study we analyzed samples from three siblings — homozygotes for the pathogenic variant c.446delT — that were reported in Ref. [40] as Family B. Surprisingly, the H₂S concentration was only raised in one asymptomatic sister from the sibship, while it was within the reference range in the other two siblings (samples from the symptomatic male index case were collected at two different occasion when he was well). In contrast, the median plasma sulfite concentration was 4.5-times increased with a substantially decreased urine concentration; a similar pattern of changes was observed for SSC. Concentrations of taurine and other sulfur compounds were within the reference range. It is unclear whether whole body homeostasis of sulfite is impaired in these patients as the data suggest a relative sparing of urinary losses with subsequently elevated plasma levels. Elevation of plasma sulfite is unlikely to be a result of increased production of cysteine sulfinic acid and sulfynilpyruvate by CDO and AST as concentrations of taurine were within the reference range. Additionally, sulfite may be released from GSSH and Cys persulfide by TST.

It is surprising that deficient activity of the first enzyme in the canonical H₂S oxidation pathway in mitochondria does not lead to a sustained increase of bioavailable sulfide levels. This raises the question whether additional pathways for H₂S catabolism may exist in humans and whether SQOR is essential for H₂S catabolism under basal physiological conditions. It is tempting to hypothesize that patients from the two SQOR deficient families (with Family B participating in the present study) have sufficient capacity to catabolize H₂S by alternative pathways, which allows them to remain clinically well, and that this capacity may have been exceeded by excessive H₂S supply from intestinal microbiota in the cases manifesting with fatal Leigh-like syndrome during childhood, without exhibiting previously major symptoms of H₂S toxicity [40].

3.3.2. Ethylmalonic encephalopathy

In agreement with previous reports [64,65], plasma sulfide was elevated in a substantial proportion of samples from EE patients, these concentrations were the highest among all disorders analyzed in this study. In EE patients, deficient PDO activity is expected to increase concentrations of GSSH, cysteine persulfide and H₂S, and to decrease production of sulfite. However, sulfite, thiosulfate, SSC and SSH were all markedly increased in plasma and/or urine whether patients were on the commonly given supplement of N-acetylcysteine or not, although those on a supplement had higher values. Accumulation of sulfite suggests increased sulfite synthesis via the pathway involving CDO and AST with cysteine sulfinic acid and sulfynilpyruvate as intermediates; this notion may be indirectly supported by the elevated taurine in urine of EE patients. Alternatively, accumulating GSSH and Cys persulfide may contribute to the sulfite pool via the activity of TST or the thioredoxin system [66]. Our results raise again novel questions on the relative roles of H₂S and sulfite accumulation in disease progression and on the present targets for therapy such as supplementation with a source of Cys and putative reduction of intestinal microbial H₂S synthesis by long-term metronidazole administration.

3.4. Patterns of metabolic changes in deficiencies of enzymes converting sulfite to sulfate

3.4.1. Sulfite oxidase and molybdenum cofactor deficiencies

Patients with SOXD, MoCD-A and MoCD-B share a similar pattern of abnormalities in sulfur-containing metabolites. We observed a two to three orders of magnitude increase in concentrations of plasma and urine sulfite as well as massive accumulation of SSC, SSH and thiosulfate in body fluids; these observations are in agreement with previous publications [10,17,67,68]. Similarly to other reports [58,59], we observed an increased urinary AASA concentration as a sign of inhibited

α -amino adipic semialdehyde dehydrogenase activity by the excess of sulfite. High chemical reactivity of sulfite is supported by direct correlation between concentrations of sulfite, and AASA, SSC and SSH in urine or plasma.

The unexpected finding of increased cystathionine concentrations in plasma and urine of some SOXD/MoCD patients does suggest impairment at the level of CSE. This can be explained by the previous observation that sulfite accumulation leads to PLP depletion, by direct inactivation and due to inhibition of α -amino adipic semialdehyde dehydrogenase [58]. Both CBS and CSE require PLP as cofactor and reduced availability of PLP will lead to secondary CSE deficiency, as seen in pyridoxine deficiency which is accompanied by cystathionine accumulation whereas CBS activity is maintained even with moderate pyridoxine deficiency [69].

Surprisingly, we have not observed any substantial decrease of plasma and urine concentrations of sulfate, the product of SOX reaction. Although we noticed an increased renal reabsorption, evidenced by decreased EF to $\approx 40\%$ of the median of controls, these data suggest that sulfite catabolism may not be the major source of sulfate in the human body and supports the notion on its major source from food [56].

As described before, tHcy and tCys in plasma were severely decreased in SOXD/MoCD patients as reported before. This can be explained by the high reactivity of sulfite with free disulfides and protein-bound cysteine and homocysteine in plasma. The resulting SSC and SSH molecules are readily excreted in urine and poorly re-absorbed as observed in this study, leading to a strong decrease of plasma tCys and tHcy.

3.5. Role of kidney in maintaining sulfur homeostasis

In addition to the above-described changes in the complex homeostatic network of enzymes that regulate metabolite fluxes, our study revealed a largely unexplored role of the kidney in maintaining sulfur concentrations in blood. Inferences on EF for tHcy and tCys cannot be made as varying proportions of their respective pools are accessible to ultrafiltration. In controls, the excretional fraction varied widely ranging from an excretion of $<5\%$ of compounds filtered from blood (e.g. taurine) to a much higher excretion $>20\%$ of filtered load for sulfate or thioethers cystathionine, lanthionine and homolanthionine. In patients, the high EF can be explained by either poor re-absorption rates (e.g. cystathionine), massively increased urinary concentrations that exceed the tubular transport maximum (e.g. thiosulfate in EE or SOXD/MoCD) or analytical artifact if total reduced thiols were measured that include disulfides that are not or very poorly re-absorbed (e.g. homocystine in CBSD). In studies of patients with inherited metabolic disorders, increased blood concentrations are typically considered markers of overproduction of the metabolite, especially if accompanied by increased urinary concentration. In this study we observed that renal tubular re-absorption appears to play an under-appreciated role in maintaining normal plasma concentrations of several metabolites (e.g. sulfate in SOXD and sulfite in SQORD), whereas it is better known that renal excretion of accumulating metabolites is an important compensatory mechanism for example with regard to SSC in SOXD and MoCD or homocystine in CBSD.

3.6. Limitations of the study

Our study has several limitations. Genetic defects in sulfur metabolism are ultra-rare diseases with high mortality and often requiring urgent treatment. Despite our best efforts it was very difficult to collect samples from patients at the time of diagnosis when not receiving any therapy. Through international collaboration we collected samples from available patients, regardless of treatment status but subsequently excluded a substantial proportion of patients on disease-modifying treatments from the final analysis to minimize the distortion of results. This increased the consistency of results but resulted in small cohorts

that preclude formal statistical analysis. Some of the observed biochemical changes however have such a large effect size that we are confident to infer on fluxes through individual pathways.

We collected samples prospectively in the majority of patients and in all controls. This was not possible for some patients in whom only stored samples were available. We have tested the stability of analytes and our data show good stability of analytes at a storage temperature of -85°C , with changes not exceeding 10% within 3 months of storage, apart from SSC and thiosulfate in plasma where a larger decrease could be observed leading to underestimation of the effect sizes (for details see Supplementary Methods SM5).

For ethical reasons we could not obtain pediatric controls and the control cohort is therefore not age-matched to patients. As in many human studies we made inferences on the whole-body homeostasis of sulfur compounds from measuring analytes in body fluids under consideration of available knowledge about their tissue distribution. Steady state plasma levels will however not allow firm conclusions on intracellular or subcellular concentrations, metabolic rates or spatial regulation.

4. Conclusions

A systematic and comprehensive assessment of metabolite concentrations in body fluids of patients with ultra-rare and severe disorders of sulfur metabolism revealed larger than expected pathobiochemical changes. In particular, sulfite accumulation had profound effects on metabolic homeostasis. Sulfite accumulation in EE or SQORD has not been described previously. We provide further evidence for new mechanisms of sulfite toxicity regarding functional pyridoxal phosphate deficiency and possible interference with CSE activity as well as preliminary evidence for a role of AOX in the metabolism of pyridoxal.

Changes in H_2S concentrations were much less pronounced than those of other metabolites and smaller than observed in many previous studies of multifactorial disease conditions that used less reliable methods for sulfide determination. There is a remarkable capacity to compensate for severe impairments of major pathways in sulfur metabolism. In this respect we provide evidence for complementary functions of CBS and CSE in H_2S synthesis and data that may potentiate the significance of the AST/MPST pathway.

Several observations in this study of human disease may have implications for clinical practice. The biochemical diagnosis of ultrarare disorders of sulfur metabolism requires highly specialized techniques, which are not readily available in practice. In contrast, tHcy assays and to a lesser extent also plasma amino acid analyses are widely available in clinical biochemical laboratories. A decrease in plasma tHcy or cysteine concentrations is often dismissed but may detect a substantial proportion of patients with sulfite intoxication disorders, including SOXD and MoCD (in the latter group with simultaneously increased SSC and decreased uric acid concentrations) while elevated tHcy is a reliable marker of severe CBSD. We propose to use tHcy or plasma amino acid analysis as the first-line test, together with a urinary dipstick test for sulfite and plasma uric acid, in patients with unexplained seizures, movement disorders and cognitive impairment, thromboembolism and lens dislocation, and to act upon finding both elevated but also decreased plasma tHcy concentrations.

Treatment outcomes for disorders in mitochondrial H_2S /sulfite catabolism is largely unsatisfactory except for liver transplant in EE [70, 71] and cyclic pyranopterin monophosphate administration in MoCD-A [72]. Our study shows that sulfite accumulates in EE and SOXD/MoCD, and that it leads to secondary disturbances in vitamin B_6 metabolism with implications for neurotransmission [10,17,73]. Two siblings with MoCD-B due to homozygous mutations in the *MOCS2* gene reported in the literature [59] had elevated urinary excretion of AASA and seizures that were responsive to treatment with pyridoxine. Our data indicate the potential for novel treatment approaches aimed at scavenging sulfite and correcting secondary vitamin B_6 abnormalities.

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Author contributions

Design of the study- VK, JS, JK, PN; collection and transport of patient and control samples, provision of clinical data and confirmation of diagnosis-BSch, BStib, HB, JC, CDV, SG, AGC, THa, THo, PJ, AK, LL, DM, FP, RS; laboratory analyses and data acquisition-JS, JK, KB, MK, TK, TVF, TD, YK; data analysis and manuscript drafting-VK, BSch, JS, PM, PC, GS, PN; manuscript revision and final approval-all authors.

Declaration of competing interest

GS declares that he serves as CEO of Colbourne Pharmaceuticals consulting Origin Biosciences in the developments of treatments for MoCD type A, BS is investigator in clinical trials to develop a treatment for MoCD-A sponsored by Origin Biosciences Inc. The other authors do not declare any competing interests.

Data availability

The data are available in the Supplementary Data file.

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Appendix A. Supplementary data

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