Published in "The FEBS Journal doi: 10.1111/febs.15214, 2020"
which should be cited to refer to this work.

The re-emerging pathophysiological role of the cystathionine-β-synthase - hydrogen sulfide system in Down syndrome

Csaba Szabo

Chair of Pharmacology, Section of Medicine, University of Fribourg, Switzerland

Keywords
bioenergetics; hydrogen sulfide; metabolism; mitochondria; trisomy

Correspondence
C. Szabo, Chair of Pharmacology, Section of Medicine, University of Fribourg, PER 17, Chemin Du Musee 18, Fribourg 1700, Switzerland
Tel: +41 26 300 9409
E-mail: csaba.szabo@unifr.ch

Down syndrome (DS) is associated with significant perturbances in many morphological and biochemical features. Cystathionine-β-synthase (CBS) is one of the key mammalian enzymes that is responsible for the biological production of the gaseous transmitter hydrogen sulfide (H_2S). When H_2S is overproduced, it can exert detrimental cellular effects, in part due to inhibition of mitochondrial Complex IV activity. An increased expression of CBS and the consequent overproduction of H_2S are well documented in individuals with DS. Two decades ago, it has been proposed that a toxic overproduction of H_2S importantly contributes to the metabolic and neurological deficits associated with DS. However, until recently, this hypothesis has not yet been tested experimentally. Recent data generated in human dermal fibroblasts show that DS cells overproduce H_2S, which, in turn, suppresses mitochondrial Complex IV activity and impairs mitochondrial oxygen consumption and ATP generation. Therapeutic CBS inhibition lifts the tonic (and reversible) suppression of Complex IV: This results in the normalization of mitochondrial function in DS cells. H_2S may also contribute to the cellular dysfunction via several other molecular mechanisms through interactions with various mitochondrial and extramitochondrial molecular targets. The current article provides a historical background of the field, summarizes the recently published data and their potential implications, and outlines potential translational approaches (such as CBS inhibition and H_2S neutralization) and future experimental studies in this re-emerging field of pathobiochemistry.

The CBS/H_2S system: emergence of a multifunctional endogenous gaseous mediator

Over the last decades, various biological regulatory roles of a novel endogenous gaseous mediator, hydrogen sulfide (H_2S), emerged (overviewed in Ref. [1–4]). These data demonstrated that H_2S is produced, in a regulated fashion, by various mammalian cells and tissues. Three major enzymes, cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) are responsible for mammalian H_2S biogenesis. CBS and CSE utilize L-cysteine and homocysteine as substrates, while 3-MST utilizes 3-mercaptopyruvate (which is also

Abbreviations
3-MST, 3-mercaptopyruvate sulfurtransferase; AOAA, aminooxyacetate; CBS, cystathionine-β-synthase; CNS, central nervous system; CSE, cystathionine-γ-lyase; DOPA, 3,4-dihydroxyphenylalanine; DS, Down syndrome; GMP, Good Manufacturing Practice; H_2S, hydrogen sulfide; O_2, oxygen; PLP, pyridoxal phosphate; RAC1, ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species.

generated from L-cysteine). H_2S serves a diverse set of biological regulatory roles in the cardiovascular, nervous, and immune system (overviewed in Ref. [1–9]). While H_2S has many distinct molecular targets and can regulate many different proteins and second messenger systems [1–9], one of the key aspects of its biological role is that it can regulate mitochondrial function. Similar to its effects on many other biological systems, its effects are bell-shaped: H_2S exerts mostly regulatory, beneficial, or protective effects at lower concentrations, and deleterious and cytotoxic effects at higher concentrations [1–13]. (The literature published prior to the mid-2000s almost exclusively focuses on the latter aspects, mostly in the context of environmental toxicology [10–13].)

At lower concentrations, H_2S stimulates mitochondrial functions and exerts protective effects in the mitochondria. As discussed elsewhere [14–17], these effects involve several different mechanisms, including direct electron donation at the level of mitochondrial Complex II, inhibition of mitochondrial protein kinase A, stimulation of mitochondrial ATP synthase (i.e., mitochondrial Complex V), formation of an antioxidant 'cloud' within the mitochondria, and stimulation of mitochondrial DNA repair via the facilitation of the assembly of mitochondrial DNA repair protein complexes. In contrast, at high concentrations, H_2S exerts deleterious effects on mitochondrial function. This effect is, to a large extent, due to a direct (but reversible) inhibition of Complex IV, a biochemical mechanism that was discovered in the 70s and has been characterized, in fine molecular detail, over the last two decades [18,19]. Complex IV inhibition, in fact, is generally accepted in the environmental toxicological literature as the primary mode of the deleterious biological actions of H_2S toxicity (which underlies the H_2S-induced metabolic suppression, the neurotoxicological effects of H_2S, and the lethal actions of severe H_2S intoxication) [10–13,20,21]. (The H_2S-mediated inhibition of mitochondrial Complex IV has many parallels of the inhibition of Complex IV by cyanide, with one important difference: The effect of cyanide is irreversible, while the effect of H_2S is reversible [19].) Most of these studies were conducted in the field of environmental toxicology, where H_2S is delivered via the inhalational route. In turn, it dissolves in the bloodstream and distributes to various cells and tissues [10–13,20,21].

The 'Kamoun Hypothesis'

Cystathionine-β-synthase is a key enzyme in the transsulfuration pathway; it catalyzes the conversion of homocysteine into cystathionine. For the last three decades, CBS has been mostly studied in the context of its inactivating mutations, which are the underlying causes of the most common inborn error of sulfur amino acid metabolism called classical homocystinuria [22,23]. Based on some 'mirror image' features evident in the clinical picture of homocystinuria vs. DS, J. Lejeune (the French physician who discovered Trisomy 21 and several other diseases related to chromosomal abnormalities) already hypothesized in 1975 [24] that the increased expression of CBS may importantly contribute to the metabolic alterations and overall clinical picture of DS.

The hypothesis that CBS-derived overproduction of H_2S may be responsible for some of the pathophysiological aspects of DS has been first put forward by P. Kamoun almost two decades ago [25], based on his own clinical findings demonstrating markedly increased circulating levels of sulfhemoglobin (a product of the reaction of hemoglobin with H_2S) and increased (approximately twofold over healthy controls) urinary excretion of thiosulfate (a stable degradation product of H_2S) in individuals with DS [26,27]. A later study, using a different H_2S detection method, has also reported increased circulating H_2S levels in DS individuals [28]. According to the 'Kamoun Hypothesis' [25], excess H_2S—at least, in part, via inhibition of mitochondrial Complex IV—may be responsible for the metabolic and neuropathological features of DS. Several lines of independent studies have demonstrated elevated expression of CBS in DS cells and tissues, including the brain [28–32]. Moreover, both the footprint of excess CBS (evidenced by low circulating homocysteine levels) [33] and the footprint of inhibition of mitochondrial oxidative phosphorylation (evidenced by the accumulation of Krebs cycle intermediaries) have been demonstrated in DS individuals by metabolomic analysis [34]. Several lines of independent studies have also demonstrated that DS cells exhibit suppressed mitochondrial function; in some (but not all) of these reports, an inhibition of Complex IV activity was noted [35–39]. (Because H_2S is a labile gaseous molecule, and the effects of H_2S on Complex IV are reversible, it is conceivable that methodological aspects have contributed to these differences: A reversible, H_2S-mediated inhibitory effect could be lifted during sample preparation and would not be expected to be readily demonstratable in Complex IV measurements in isolated mitochondrial or mitochondrial fractions.) However, whether endogenous H_2S overproduction is directly responsible for the suppression of mitochondrial function in DS has not been directly tested until recently.

Experimental confirmation of the Kamoun Hypothesis

When the Kamoun Hypothesis was originally proposed (almost two decades ago), it may have been considered 'wild' or 'far out': H_2S, at the time, was not generally accepted as an endogenous mammalian biological mediator or regulator. However, work in the last two decades has demonstrated the multiple biological roles of this mediator [1–9] and started to distinguish between 'high-H_2S' pathophysiological states (e.g., certain forms of inflammation and shock) and 'low-H_2S' pathophysiological states (e.g., various forms of vascular diseases) [9]. Thus, the field of H_2S biology has expanded and matured to enable revisiting the Kamoun Hypothesis. Also, the experimental tools needed to address the Hypothesis became more sophisticated. For instance, instead of studying Complex IV activity in isolated mitochondria or in mitochondrial fractions (which methods are particularly limited to study the effects of labile, diffusible mediators such as H_2S), now it is possible to study the activity of Complex IV in whole cells, using, for instance, the extracellular flux analysis method. Using this method in permeabilized dermal fibroblast from DS cells, the potential role of H_2S overproduction was recently revisited in the pathogenesis of mitochondrial dysfunction [40]. The study was based on the comparison of dermal fibroblasts from a female individual with DS (Detroit 539) with an age-matched healthy female subject (Detroit 551) (control cells) *in vitro*. The results confirmed that CBS protein levels, as well as H_2S cellular levels, are markedly elevated in DS cells. The localization of CBS in DS cells was both cytosolic and mitochondrial. Extracellular flux analysis of DS cells demonstrated a profound suppression of mitochondrial electron transport, mitochondrial oxygen (O_2) consumption, and ATP generation, which has also resulted in a suppressed proliferation rate. Measurement of the specific activity of Complex IV revealed a profound inhibition of Complex IV activity in DS cells [40]. Pharmacological inhibition of CBS using aminooxyacetate (AOAA), a small molecule that interacts with the pyridoxal phosphate (PLP) prosthetic group in the active center of the enzyme [41], restored Complex IV activity and improved mitochondrial electron transport and cell proliferation, and similar effects were also observed when in DS cells CBS expression was normalized by siRNA [40]. In contrast, in normal control cells, CBS inhibition or CBS silencing did not enhance mitochondrial function. However, a suppression of mitochondrial function could be produced in control cells by exposing them to a pharmacological H_2S donor; the same donor molecule also reversed the mitochondrial stimulatory effects of CBS inhibition in DS cells [40].

The above findings can be conceptualized on a bell-shaped curve that represents the pharmacological effects of low vs. high levels of H_2S in terms of mitochondrial function and cell viability (Fig. 1): in DS cells, high intracellular H_2S levels induce Complex IV blockade, suppress cellular bioenergetics, and impair proliferation. Because the H_2S-mediated inhibition of Complex IV is a reversible biochemical process, pharmacological inhibition of CBS-dependent H_2S production normalizes these responses. In contrast, in control cells, low endogenous levels of H_2S either stimulate bioenergetics or do not affect it in a significant manner; in this situation, pharmacological inhibition of CBS either suppresses or does not significantly affect mitochondrial function, while excess H_2S in control cells (through delivery with the H_2S donor) phenocopies the suppression of mitochondrial function and cell proliferation seen in DS cells.

Neuronal CBS overexpression and H_2S neurotoxicity contributes to DS-associated neuronal disturbances

Overproduction of H_2S and the resultant inhibition of mitochondrial Complex IV may well explain some of the neurological and neurocognitive deficits associated with DS. Neurons are metabolically highly active; they are highly dependent on ATP produced by oxidative phosphorylation [42]. Nevertheless, the study referenced in the previous section [40] only utilized fibroblasts and not neurons; therefore, direct proof of this hypothesis remains to be conducted.

Nevertheless, it is well known that neurons are highly sensitive to H_2S exposure and H_2S is a well-known neurotoxin that appears to exert a significant part of its toxicological action through Complex IV inhibition; after exposure of animals to toxicological doses of H_2S, an inhibition of mitochondrial Complex IV was reported in the brains of the animals *ex vivo* [10,11,21]. (Because the effect of H_2S on Complex IV is a reversible biochemical process, we can assume that the degree of this inhibition *in vivo* was, in fact, more pronounced than the degree of inhibition detected in the *ex vivo* assay). Environmental toxicology studies demonstrate, for instance, that chronic exposure of pregnant animals to inhaled H_2S produces significant neurodevelopmental alterations. In one study, exposure to relatively low concentrations of inhaled H_2S to time-pregnant rats (between day 5 postcoitus and day

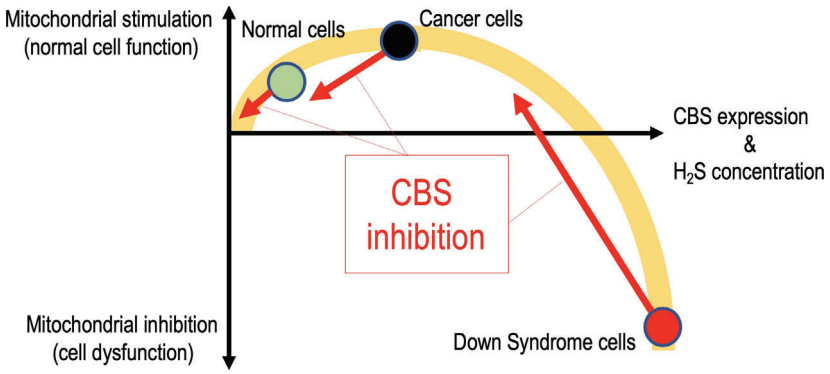

Fig. 1. Role of low and high levels of H_2S on mitochondrial function in control cells, in certain cancer cells, and in DS cells. The *x*-axis denotes increasing expression of CBS and corresponding increasing intracellular concentrations of H_2S. The y-axis denotes stimulation or inhibition of mitochondrial function. In normal cells, the lower endogenous levels of H_2S do not affect mitochondrial function in a significant manner. Therefore, inhibition of CBS (red arrow) does not affect this parameter in a significant manner. In various forms of cancer, CBS and H_2S are upregulated to an extent where they serve biological purposes. In these conditions, inhibition of H_2S biosynthesis (red arrow) suppresses mitochondrial function. In DS cells, high intracellular H_2S levels induce Complex IV blockade and suppress mitochondrial function. In these conditions, inhibition of H_2S biosynthesis (red arrow) restores the suppression of mitochondrial function.

21 postnatal) produces detectable architectural modifications of cerebellar Purkinje cells, alteration of various amino acid neurotransmitters, and changes in monoamine levels in the developing rat brain [43,44]. In addition, chronic exposure to low concentrations of H_2S was found to produce abnormal growth in developing cerebellar Purkinje cells [45]. Moreover, chronic H_2S inhalation was also reported to impair learning and memory performance in adult rats [46]. Importantly, a partially overlapping set of neurological disturbances—for example, altered hippocampal long-term potentiation, dysregulation of brain serotonin and dopamine pathways, and impaired neurocognitive parameters, such as impaired performance on the novel object recognition test—were observed in transgenic mice that overexpress CBS in their brain [47–49]. Importantly, normalization of CBS expression in an animal model of DS (DP1Yah mice, which contain an extra copy of mouse chromosome 17 that encodes murine CBS—as well as a number of additional genes) improved the locomotor and neurocognitive deficit that is characteristic to these animals [49]. Similar neurological improvements were also found when animals were treated with disulfiram, a compound that may act by normalizing some of the cellular dysfunction elicited by the excess CBS in the neurons [49].

All of the above findings are consistent with the hypothesis that the excess CBS in the neurons, and the consequent, chronic exposure to neurotoxic levels of endogenously produced H_2S contributes to the neurocognitive defects associated with DS. Direct studies (e.g., using selective, direct pharmacological CBS inhibitors, or H_2S scavengers; see below) remain to be conducted to directly test this hypothesis. It is also unknown at present, whether the H_2S system in particular (or, perhaps, mitochondrial and bioenergetic deficits, in general) may contribute to the development of Alzheimer-type pathologies that individuals with DS develop later in their lives.

Potential additional roles of the CBS/H_2S system in DS

If the CBS/H_2S-mediated suppression of mitochondrial Complex IV activity and, consequently, a suppression of cellular bioenergetics is, indeed, a generalized phenomenon that occurs in all (or most) cells and tissues of individuals with DS, this would offer a potential explanation for many characteristic features of DS, not only the neurocognitive defects (discussed in the prior section), but also other pathophysiological features.

For instance, an H_2S-induced impairment of mitochondrial function and a resulting impairment of aerobic ATP generation may well explain the muscle hypotonia and the low aerobic fitness that is observed in individuals with DS. It is, indeed, well documented that individuals with DS are known to have an impaired muscle strength and extremely low maximal aerobic capacity [36,50,51].

Individuals with DS are protected from many types of cancer, and they also have a markedly reduced incidence to develop atherosclerosis [52]. While the mechanism behind the protection against cancer may, in theory, involve some mechanism that may be related to the cytoprotective aspects of H_2S, there are

currently no published studies in this regard, and we cannot speculate on this aspect of the condition. However, with respect to the cardiovascular protective phenotype, it may well be that the excess circulating H_2S may be a factor that contributes to this protective effect. In fact, the potential role of the CBS system in counteracting atherosclerosis in individuals with DS has been put forward as early as the early 90s [52]. Indeed, there are many studies demonstrating that H_2S donation can, in fact, provide a marked protective effect against the development of atherosclerosis in various experimental models [53–55]. DS individuals also tend to have a low systemic blood pressure [56]; it is interesting, in this context, that H_2S is an endogenous mediator that can cause vascular relaxation to decrease blood pressure [1–9].

Potential avenues for therapeutic modulation of the CBS/H_2S system in DS

There are currently no clinically applicable pharmacological CBS inhibitors, and it is unlikely that dietary or lifestyle changes could meaningfully reduce impact the CBS-linked H_2S overproduction in individuals with DS. The most commonly used (gold standard) CBS inhibitor is AOAA [9]. This molecule has a low micromolar half-maximal inhibitory concentration in human CBS [41], but it has a low cell penetration and its cell-based potency is relatively low, although there may be ways to improve its cell uptake by producing cell-permeable AOAA prodrugs [9,41,57]. Nevertheless, inhibition of CBS has been successfully achieved *in vivo* in various animal models associated with CBS-related H_2S overproduction [58–60], including a study of chronic alcoholism-induced learning and memory dysfunction, where the inhibitor, in fact, has been found to exert beneficial effects [60]. (Interestingly, this study reported that the alcoholism model was associated with an overproduction of H_2S, which was, in turn, normalized by AOAA treatment in these animals.) Four decades ago, AOAA has been clinically tested in small-scale investigator-initiated studies in patients with Huntington's disease and tinnitus [61,62]. In these studies, the compound was relatively well tolerated (but its therapeutic efficacy was unproven). It should be noted that these clinical studies were conducted in an era where the regulatory requirements were more relaxed (i.e., the clinical study was not preceded by formal Good Laboratory Practice preclinical animal safety and toxicology studies, and the trials did not use Good Manufacturing Practice (GMP)-manufactured material or a pharmaceutically acceptable drug formulation). The information that can be gained from these studies is also limited by the fact that AOAA plasma levels or metabolites were not measured and AOAA's oral bioavailability was not established. It must be mentioned that AOAA's selectivity as a CBS inhibitor is rather limited: The compound inhibits not only CBS, but also a second H_2S-producing enzyme, CSE, as well as a variety of other enzymes that contain a PLP prosthetic group [9,41]. (In addition to CBS and CSE, AOAA is known to inhibit several other enzymes including aspartate transaminase and gamma-aminobutyric acid transaminase.) Moreover, AOAA is an irreversible enzyme inhibitor; irreversible inhibitors are generally less favored as therapeutic agents than reversible inhibitors. (Even though there are, in fact, many clinically used drugs that exert irreversible inhibitory effects on their target, for example, aspirin is an irreversible inhibitor of cyclooxygenase.) Clearly, for future clinical trials targeting the CBS pathway in DS, a novel, potent, highly selective, cell-permeable, and pharmaceutically acceptable CBS inhibitor would be preferable to AOAA.

A potentially repurposable CBS inhibitor, identified by a physical screening campaign on human recombinant CBS [63], is the clinically used 3,4-dihydroxyphenylalanine (DOPA) decarboxylase inhibitor benserazide, which—similar to AOAA, exerts its effect via a PLP-dependent inhibitory action. Interestingly, this molecule was found to elevate homocysteine levels in patients [64], which is consistent with an inhibitory action on CBS. However, as discussed by P. Kamoun [65], benserazide has poor central nervous system (CNS) penetration, and it would only be potentially suitable to counteract the peripheral, but not the central aspects of CBS-mediated H_2S overproduction in DS. Benserazide is actually not a stand-alone drug; it is formulated as a component of a two-component therapeutic agent, madopar (benserazide + L-DOPA) in many European countries and Canada. (Any future effort to re-introduce AOAA into clinical trials would be viewed, by the regulatory agencies, as a 'new chemical entity' project; that is, any renewal of clinical studies utilizing AOAA would have to be supported by a new set of formal safety and toxicology studies, and the compound would have to be chemically synthetized under GMP processes.)

There are several reports identifying, through various *in silico* modeling and/or physical screening efforts, several other CBS inhibitor small molecules [9,66,67]; however, the characteristics of these compounds are incompletely understood: They can currently only be viewed as experimental tools, rather than potential clinical development candidates.

In addition to pharmacological inhibitors of CBS, theoretical possibilities to counteract H_2S intoxication *in vivo* include various forms of H_2S scavenging. Potential scavengers include sodium nitrite, vitamin B12a (hydroxocobalamin), vitamin B12 analogs (e.g., cobamamide and dinitrocobamamide), and methylene blue [reviewed in Ref. [13,65]]. Scavengers, in general, are less elegant (and, more importantly, less effective) than direct inhibition of the enzymatic target at the source. Nevertheless, some of these scavengers are already used in patients: Sodium nitrite is clinically used as an antidote for acute cyanide poisoning—although it is typically formulated together with thiosulfate, that is, a stable degradation product of H_2S, which, however, in some cases can also be metabolized back to H_2S [68]. In addition, oral hydroxocobalamin formulations are available as nutritional supplements. There is also an intravenous form of this molecule, approved for patients as a cyanide intoxication antidote (Cyanokit). Some of the above listed H_2S scavengers may be amenable for investigator-initiated clinical studies.

Caveats, remaining questions, and potential future directions

Down syndrome is associated with the dysregulation of over 600 genes and gene products, the majority (over ¾) of which are not even encoded on chromosome 21 [69–72]. These findings suggest a global dysregulation of gene expression in DS, which may be mediated, at least in part, by changes in histone acetylation [70]. Undoubtedly, there is a profound dysregulation of multiple key cellular signaling, metabolic, immunologic, and other regulatory processes in all cells and tissues of DS individuals. How, then, could one single protein (CBS), one single enzymatic product (H_2S), and one single biochemical mechanism (inhibition of Complex IV) play a crucial role in the pathophysiology of DS? Furthermore, if, indeed, Complex IV is profoundly suppressed in all cells and tissues in DS, would such a severe biochemical alteration be compatible with fundamental cellular and physiological functions?

While complete answers do not exist to the above questions, some points can be highlighted. First of all, clearly, there are many different signaling and metabolic disturbances in DS. In mitochondria alone, many different disturbances have been reported, ranging from changes in the expression and/or activity of many mitochondrial electron transport complexes, changes in the organization, size, and shape of the mitochondria, alterations in mitochondrial calcium handling, impairment of mitochondrial DNA integrity, enhanced mitochondrial reactive oxygen species (ROS) generation, and many others [35–40]. On the one hand, it is conceivable that the Complex IV inhibition-related mechanism occurs on the background of dysfunctional disturbed mitochondrion, which may therefore become more sensitive to a H_2S-mediated inhibition of Complex IV (and consequent shutdown of electron transport and suppression of ATP synthesis) than a 'normal' mitochondrion. On the other hand, it is also conceivable (but remains to be experimentally tested) that some of the mitochondrial disturbances listed above are the consequences of Complex IV inhibition. For instance, if Complex IV is blocked, electrons can 'back up' and 'leak off' from mitochondrial complexes II or III which, in turn, may result in increased ROS formation. Increased mitochondrial ROS production is known to induce mitochondrial DNA damage, because mitochondrial DNA is known to be extremely sensitive to oxidative damage (much more so than nuclear DNA) [17,73]. Mitochondrial DNA damage, and subsequent dysregulation of local (intramitochondrial) protein synthesis, in turn, may well induce additional functional and potential morphological alterations.

It is also conceivable that the pathophysiological disturbances created by H_2S in DS involve not only Complex IV, but also many other molecular targets (Table 1). H_2S, as a lipophilic, diffusible mediator, can travel from one cellular compartment to another or from one cell to another. It may cause changes in gene expression, and it may influence the activity of many proteins and enzymes. A significant part of the latter processes occurs through a global post-transcriptional regulatory mechanism termed sulfhydration (modification of critical cysteines in enzymes). Some of the sulfhydration reactions occur through polysulfide chemistry (and not H_2S *per se*) [74,75], but increased cellular H_2S and polysulfide generation usually occurs hand in hand in various pathophysiological states. In this context, it is important to mention that DS cells were reported to have a markedly elevated intracellular polysulfide content compared to normal control cells [40]. H_2S also has many other molecular targets: For instance, it can alter the activity of various membrane channels, and it can inhibit carbonic anhydrase and monoamine oxidase [9,25]. Inhibition of monoamine oxidase would be expected to create disturbances in neurotransmitters in the nervous system in DS. Marechal and coworkers have hypothesized that H_2S overproduction in DS may activate ras-related C3 botulinum toxin substrate 1 (RAC1), which, in turn, could lead to the rearrangement of the actin cytoskeleton, thereby creating disturbances in synaptic transmission [49]. Clearly, there are many potential

Table 1. Confirmed and potential biochemical targets and biological effects of excess H_2S in DS.

Confirmed targets of H_2S	Biochemical effect	Consequences/ potential consequences in DS
Mitochondrial Complex IV	Reversible, tonic inhibition	Inhibition of electron transport, impairment of aerobic ATP generation, suppression of cellular bioenergetics. These could potentially lead to increased mitochondrial ROS production and mitochondrial DNA damage
Hemoglobin	Sulfhemoglobin formation	Decreased O_2 carrying ability of red blood cells, diminished O_2 transport to the tissues, reduced tissue metabolism
Potential targets of H_2S	**Biochemical effect**	**Potential consequences in DS**
Cysteine residues in various proteins	Formation of -SSH groups, resulting in increased or decreased protein function	Depending on the target protein's biological role, many cellular functions can be affected, such as cell signaling, cell death, and metabolism. This can, in turn, adversely affect neurotransmission, neuronal development, and many other biological functions
DNA	DNA strand breakage and oxidative DNA base modification (via increased ROS generation and/or inhibition of DNA repair enzymes)	Mutations, micronucleus formation, promotion of certain types of cancer, cell cycle arrest, developmental and global cellular defects
Promoter systems and histones	Modulation of promoter activity and/or histone deacetylase activity, leading to dysregulation of chromatin remodeling	Global dysregulation of gene expression, global cellular and developmental effects
Na+/K + ATPase	Inhibition of enzymatic activity	Cellular bioenergetic defects, metabolic dysfunction
Carbonic anhydrase	Inhibition of enzymatic activity	Dysregulation of acid–base balance, disturbed transport of carbon dioxide away from tissues during respiration, renal impairment, dysregulation of neuronal lipid biosynthesis, altered organization of myelin membranes
Monoamine oxidase	Inhibition of enzymatic activity	Increased CNS catecholamine and serotonin levels, neuronal dysfunction, impairment of neuronal development
Acetylcholinesterase	Inhibition of enzymatic activity	Increased CNS acetylcholine levels, disturbed synaptic transmission
RAC1	Activation	Disturbances of synaptic vesicle release, inhibition of neurotransmitter release
Potassium, calcium, and other membrane channels	Activation or inhibition	Disturbances in membrane potential (depolarization or hyperpolarization), global dysregulation of cell function
Sodium-dependent transport of L-glutamate in synaptosomes	Inhibition	Dysregulation of neuronal function
N-methyl-D-aspartic acid receptors	Activation due to increased CNS glutamate levels	Excitotoxicity, dysregulation of neuronal function
Nuclear transcription factors	Increased gene transcription, inflammatory mediator production	Inflammatory mediator imbalance, immune system defects
Immune cells	Dysregulation of immune function through various mechanisms	Immune system disturbances
Transient receptor potential ankyrin 1, T-type calcium channel, Cav3.2 isoform	Activation and induction of pronociceptive effects	Increased pain sensitivity
Vascular cGMP system	Increased cGMP levels	Hypotension, protection against atherosclerosis or against cardiovascular dysfunction

mechanisms that remain to be studied as far as the pathophysiological targets of H_2S in DS are concerned. There may also be compensatory mechanisms (mitochondrial or, perhaps, related to the regulation or CBS through allosteric mechanism, or potentially compensatory changes in various enzymes affecting

H_2S catabolism) in DS. The potential role of such compensatory systems remains to be explored.

It is likely that the relative role of H_2S in DS depends on the cell type, the organ, the stage of the condition, and perhaps even the type of DS (there are many cases of partial chromosome 21 trisomy, which yield variable clinical pictures). There may well be a 'point-of-no-return' in DS, where the various CBS/H_2S-mediated processes produce cellular disturbances that may no longer be reversible even after H_2S overproduction is pharmacologically corrected. All of the above issues remain to be addressed in future studies; such work may, nevertheless, go hand in hand with well-considered translational and experimental therapeutic approaches to target the CBS/H_2S pathway in DS.

Acknowledgements

The work of CS in the area of Down syndrome is supported by a grant from the LeJeune Foundation (Paris).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

CS conceived and wrote the manuscript.

References

1 Li L, Rose P & Moore PK (2011) Hydrogen sulfide and cell signaling. *Ann Rev Pharmacol Toxicol* **51**, 169–187.
2 Wang R (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol Rev* **92**, 791–896.
3 Kimura H (2014) Production and physiological effects of hydrogen sulfide. *Antioxid Redox Signal* **20**, 783–793.
4 Rose P, Moore PK & Zhu YZ (2017) H_2S biosynthesis and catabolism: new insights from molecular studies. *Cell Mol Life Sci* **74**, 1391–1412.
5 Tan BH, Wong PT & Bian JS (2010) Hydrogen sulfide: a novel signaling molecule in the central nervous system. *Neurochem Int* **56**, 3–10.
6 Paul BD & Snyder SH (2012) H_2S signalling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol* **13**, 499–507.
7 Polhemus DJ & Lefer DJ (2014) Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. *Circ Res* **114**, 730–737.
8 Szabo C (2017) Hydrogen sulfide, an enhancer of vascular nitric oxide signaling: mechanisms and implications. *Am J Physiol Cell Physiol* **312**, C3–C15.
9 Szabo C & Papapetropoulos A (2017) International Union of Basic and Clinical Pharmacology. CII: pharmacological modulation of H_2S levels: H_2S donors and H_2S biosynthesis inhibitors. *Pharmacol Rev* **69**, 497–564.
10 Beauchamp RO, Bus JS, Popp JA, Boreiko CJ & Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. *CRC Crit Rev Toxicol* **13**, 25–97.
11 Reiffenstein RJ, Hulbert WC & Roth SH (1992) Toxicology of hydrogen sulfide. *Ann Rev Pharmacol Toxicol* **32**, 109–134.
12 Szabo C (2018) A timeline of hydrogen sulfide (H_2S) research: from environmental toxin to biological mediator. *Biochem Pharmacol* **149**, 5–19.
13 Ng PC, Hendry-Hofer TB, Witeof AE, Brenner M, Mahon SB, Boss GR, Haouzi P & Bebarta VS (2019) Hydrogen sulfide toxicity: mechanism of action, clinical presentation, and countermeasure development. *J Med Toxicol* **15**, 287–294.
14 Pun PB, Lu J, Kan EM & Moochhala S (2010) Gases in the mitochondria. *Mitochondrion* **10**, 83–93.
15 Szabo C, Ransy C, Módis K, Andriamihaja M, Murghes B, Coletta C, Olah G, Yanagi K & Bouillaud F (2014) Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol* **171**, 2099–2122.
16 Módis K, Bos EM, Calzia E, van Goor H, Coletta C, Papapetropoulos A, Hellmich MR, Radermacher P, Bouillaud F & Szabo C (2014) Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II. Pathophysiological and therapeutic aspects. *Br J Pharmacol* **171**, 2123–2146.
17 Szczesny B, Marcatti M, Zatarain JR, Druzhyna N, Wiktorowicz JE, Nagy P, Hellmich MR & Szabo C (2016) Inhibition of hydrogen sulfide biosynthesis sensitizes lung adenocarcinoma to chemotherapeutic drugs by inhibiting mitochondrial DNA repair and suppressing cellular bioenergetics. *Sci Rep* **6**, 36125.
18 Chance B & Schoener B (1965) High and low energy states of cytochromes. I. In mitochondria. *J Biol Chem* **241**, 4567–4573.
19 Nicholls P, Marshall DC, Cooper CE & Wilson MT (2013) Sulfide inhibition of and metabolism by cytochrome c oxidase. *Biochem Soc Trans* **41**, 1312–1316.
20 Nicholson RA, Roth SH & Jian Zheng AZ (1998) Inhibition of respiratory and bioenergetic mechanisms by hydrogen sulfide in mammalian brain. *J Toxicol Environment Health* **54**, 491–507.
21 Dorman DC, Moulin FJM, McManus BE, Mahle KC, James RA & Struve MF (2002) Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat

brain, liver, lung, and nasal epithelium. *Toxicol Sci* **65**, 18–25.
22 Sacharow SJ, Picker JD & Levy HL (2004) Homocystinuria caused by cystathionine beta-synthase deficiency. In GeneReviews® (Adam MP, Ardinger HH & Pagon RA *et al*., eds) University of Washington, Seattle, WA; 1993-2019. [Updated 2017 May 18].
23 Majtan T, Pey AL, Gimenez-Mascarell P, Martínez-Cruz LA, Szabo C, Kožich V & Kraus JP (2018) Potential pharmacological chaperones for cystathionine beta-synthase-deficient homocystinuria. *Handb Exp Pharmacol* **245**, 345–383.
24 Lejeune J (1975) Réflexions sur la débilité de l' intelligence des enfants trisomiques 21. *Pont Acad Sci* **3**, 1–12.
25 Kamoun P (2001) Mental retardation in Down syndrome: a hydrogen sulfide hypothesis. *Med Hypotheses* **57**, 389–392.
26 Belardinelli MC, Chabli A, Chadefaux-Vekemans B & Kamoun P (2001) Urinary sulfur compounds in Down syndrome. *Clin Chem* **47**, 1500–1501.
27 Kamoun P, Belardinelli MC, Chabli A, Lallouchi K & Chadefaux-Vekemans B (2003) Endogenous hydrogen sulfide overproduction in Down syndrome. *Am J Med Genet* **116A**, 310–311.
28 Abdel-Salam E, Abdel-Meguid I & Korraa S (2013) Assessment of immune function in Down syndrome patients. *Egyptian J Med Hum Genet* **14**, 307–310.
29 Chadefaux B, Rethoré MO, Raoul O, Ceballos I, Poissonnier M, Gilgenkranz S & Allard D (1985) Cystathionine beta synthase: gene dosage effect in trisomy 21. *Biochem Biophys Res Commun* **128**, 40–44.
30 Ge Y, Jensen TL, Matherly LH & Taub JW (2003) Transcriptional regulation of the cystathionine-beta - synthase gene in Down syndrome and non-Down syndrome megakaryocytic leukemia cell lines. *Blood* **101**, 1551–1557.
31 Ichinohe A, Kanaumi T, Takashima S, Enokido Y, Nagai Y & Kimura H (2005) Cystathionine beta-synthase is enriched in the brains of Down's patients. *Biochem Biophys Res Commun* **338**, 1547–1550.
32 Pinilla JM, Ayala-Ramírez P, García-Robles R, Olaya-C M & Bermúdez M (2015) Expression of cystathionine beta-synthase and histopathological observations in placentas of patients with Down syndrome. *J Neonatal Perinatal Med* **8**, 77–84.
33 Pogribna M, Melnyk S, Pogribny I, Chango A, Yi P & James SJ (2001) Homocysteine metabolism in children with Down syndrome: *in vitro* modulation. *Am J Hum Genet* **69**, 88–95.
34 Caracausi M, Ghini V, Locatelli C, Mericio M, Piovesan A, Antonaros F, Pelleri MC, Vitale L, Vacca RA, Bedetti F *et al*. (2018) Plasma and urinary metabolomic profiles of Down syndrome correlate with alteration of mitochondrial metabolism. *Sci Rep* **8**, 2977.
35 Prince J, Jia S, Båve U, Annerén G & Oreland L (1994) Mitochondrial enzyme deficiencies in Down's syndrome. *J Neural Transm Park Dis Dement Sect* **8**, 171–181.
36 Cowley PM, Keslacy S, Middleton FA, DeRuisseau LR, Fernhall B, Kanaley JA & DeRuisseau KC (2012) Functional and biochemical characterization of soleus muscle in Down syndrome mice: insight into the muscle dysfunction seen in the human condition. *Am J Physiol Regul Integr Comp Physiol* **303**, R1251–R1260.
37 Piccoli C, Izzo A, Scrima R, Bonfiglio F, Manco R, Negri R, Quarato G, Cela O, Ripoli M, Prisco M *et al*. (2013) Chronic pro-oxidative state and mitochondrial dysfunctions are more pronounced in fibroblasts from Down syndrome foeti with congenital heart defects. *Hum Mol Genet* **22**, 1218–1232.
38 Zamponi E & Helguera PR (2019) The shape of mitochondrial dysfunction in Down Syndrome. *Dev Neurobiol* **79**, 613–621.
39 Izzo A, Mollo N, Nitti M, Paladino S, Calì G, Genesio R, Bonfiglio F, Cicatiello R, Barbato M, Sarnataro V *et al*. (2018) Mitochondrial dysfunction in Down syndrome: molecular mechanisms and therapeutic targets. *Mol Med* **24**, 2.
40 Panagaki T, Randi EB, Augsburger F & Szabo C (2019) Overproduction of H2S, generated by CBS, inhibits mitochondrial Complex IV and suppresses oxidative phosphorylation in Down syndrome. *Proc Natl Acad Sci USA* **116**, 18769–18771.
41 Asimakopoulou A, Panopoulos P, Chasapis CT, Coletta C, Zhou Z, Cirino G, Giannis A, Szabo C, Spyroulias GA & Papapetropoulos A (2013) Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). *Br J Pharmacol* **169**, 922–932.
42 Hall CN, Klein-Flügge MC, Howarth C & Attwell D (2012) Oxidative phosphorylation, not glycolysis, powers presynaptic and postsynaptic mechanisms underlying brain information processing. *J Neurosci* **32**, 8940–8951.
43 Roth SH, Skrajny B & Reiffenstein RJ (1995) Alteration of the morphology and neurochemistry of the developing mammalian nervous system by hydrogen sulphide. *Clin Exp Pharmacol Physiol* **22**, 379–380.
44 Skrajny B, Hannah RS & Roth SH (1992) Low concentrations of hydrogen sulphide alter monoamine levels in the developing rat central nervous system. *Can J Physiol Pharmacol* **70**, 1515–1518.
45 Partlo LA, Sainsbury RS & Roth SH (2001) Effects of repeated hydrogen sulphide (H_2S) exposure on learning and memory in the adult rat. *Neurotoxicology* **22**, 177–189.
46 Hannah RS & Roth SH (1991) Chronic exposure to low concentrations of hydrogen sulfide produces abnormal growth in developing cerebellar Purkinje cells. *Neurosci Lett* **122**, 225–228.

47 Régnier V, Billard JM, Gupta S, Potier B, Woerner S, Paly E, Ledru A, David S, Luilier S, Bizot JC *et al.* (2012) Brain phenotype of transgenic mice overexpressing cystathionine β-synthase. *PLoS ONE* **7**, e29056.
48 London J, Ndiaye FK, Bui LC, Souchet B, Daubigney F, Magnan C, Luquet S, Dairou J, Janel N & Rouch C (2019) Alterations in the serotonin and dopamine pathways by cystathionine beta synthase overexpression in murine brain. *Mol Neurobiol* **56**, 3958–3971.
49 Marechal D, Brault V, Leon A, Martin D, Lopes Pereira P, Loaëc N, Birling MC, Friocourt G, Blondel M & Herault Y (2019) Cbs overdosage is necessary and sufficient to induce cognitive phenotypes in mouse models of Down syndrome and interacts genetically with Dyrk1a. *Hum Mol Genet* **28**, 1561–1577.
50 Pitetti KH & Boneh S (1995) Cardiovascular fitness as related to leg strength in adults with mental retardation. *Med Sci Sports Exerc* **27**, 423–428.
51 Carmeli E, Barchad S, Lenger R & Coleman R (2002) Muscle power, locomotor performance and flexibility in aging mentally-retarded adults with and without Down's syndrome. *J Musculoskelet Neuronal Interact* **2**, 457–462.
52 Nordström M & Kjellström T (1992) Age dependency of cystathionine beta-synthase activity in human fibroblasts in homocyst(e)inemia and atherosclerotic vascular disease. *Atherosclerosis* **94**, 213–221.
53 Mani S, Li H, Untereiner A, Wu L, Yang G, Austin RC, Dickhout JG, Lhoták Š, Meng QH & Wang R (2013) Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation* **127**, 2523–2534.
54 Liu Z, Han Y, Li L, Lu H, Meng G, Li X, Shirhan M, Peh MT, Xie L, Zhou S *et al.* (2013) The hydrogen sulfide donor, GYY4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein $E^{-/-}$ mice. *Br J Pharmacol* **169**, 1795–1809.
55 Bibli SI, Hu J, Sigala F, Wittig I, Heidler J, Zukunft S, Tsilimigras DI, Randriamboavonjy V, Wittig J, Kojonazarov B *et al.* (2019) Cystathionine γ lyase sulfhydrates the RNA binding protein Human Antigen R to preserve endothelial cell function and delay atherogenesis. *Circulation* **139**, 101–114.
56 Morrison RA, McGrath A, Davidson G, Brown JJ, Murray GD & Lever AF (1996) Low blood pressure in Down's Syndrome. A link with Alzheimer's Disease? *Hypertension* **28**, 569–575.
57 Chao C, Zatarain JR, Ding Y, Coletta C, Mrazek AA, Druzhyna N, Johnson P, Chen H, Hellmich JL, Asimakopoulou A *et al.* (2016) Cystathionine-beta-synthase inhibition for colon cancer: enhancement of the efficacy of aminooxyacetic acid via the prodrug approach. *Mol Med* **22**, 361–379.
58 Roy A, Khan AH, Islam MT, Prieto MC & Majid DS (2012) Interdependency of cystathione γ-lyase and cystathionine β-synthase in hydrogen sulfide-induced blood pressure regulation in rats. *Am J Hypertens* **25**, 74–81.
59 Szabo C, Coletta C, Chao C, Módis K, Szczesny B, Papapetropoulos A & Hellmich MR (2013) Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. *Proc Natl Acad Sci USA* **110**, 12474–12479.
60 Du AL, Qin HZ, Jiang HB, Fu PY, Lou K & Xu YM (2018) Aminooxyacetic acid improves learning and memory in a rat model of chronic alcoholism. *Neural Regen Res* **13**, 1568–1574.
61 Perry TL, Wright JM, Hansen S, Allan BM, Baird PA & MacLeod PM (1980) Failure of aminooxyacetic acid therapy in Huntington disease. *Neurology* **30**, 772–775.
62 Guth PS, Risey J, Briner W, Blair P, Reed HT, Bryant G, Norris C, Housley G & Miller R (1990) Evaluation of amino-oxyacetic acid as a palliative in tinnitus. *Ann Otol Rhinol Laryngol* **99**, 74–79.
63 Druzhyna N, Szczesny B, Olah G, Módis K, Asimakopoulou A, Pavlidou A, Szoleczky P, Gerö D, Yanagi K, Törö G *et al.* (2016) Screening of a composite library of clinically used drugs and well-characterized pharmacological compounds for cystathionine β-synthase inhibition identifies benserazide as a drug potentially suitable for repurposing for the experimental therapy of colon cancer. *Pharmacol Res* **113**, 18–37.
64 Guo G, Xu S, Cao LD & Wu QY (2016) The effect of levodopa benserazide hydrochloride on homocysteinemia levels in patients with Parkinson's disease and treatment of hyperhomocysteinemia. *Eur Rev Med Pharmacol Sci* **20**, 2409–2412.
65 Kamoun PP (2019) Mental retardation in Down syndrome: two ways to treat. *Med Hypotheses* **131**, 109289.
66 Niu W, Chen F, Wang J, Qian J & Yan S (2017) Antitumor effect of sikokianin C, a selective cystathionine β-synthase inhibitor, against human colon cancer *in vitro* and *in vivo*. *Medchemcomm* **9**, 113–120.
67 Wang L, Cai H, Hu Y, Liu F, Huang S, Zhou Y, Yu J, Xu J & Wu F (2018) A pharmacological probe identifies cystathionine β-synthase as a new negative regulator for ferroptosis. *Cell Death Dis* **9**, 1005.
68 Olson KR, Deleon ER, Gao Y, Hurley K, Sadauskas V, Batz C & Stoy GF (2013) Thiosulfate: a readily accessible source of hydrogen sulfide in oxygen sensing. *Am J Physiol Regul Integr Comp Physiol* **305**, 592–603.
69 Sullivan KD, Lewis HC, Hill AA, Pandey A, Jackson LP, Cabral JM, Smith KP, Liggett LA, Gomez EB, Galbraith MD *et al.* (2016) Trisomy 21 consistently activates the interferon response. *Elife* **5**, e16220.

70 Mowery CT, Reyes JM, Cabal-Hierro L, Higby KJ, Karlin KL, Wang JH, Kimmerling RJ, Cejas P, Lim K, Li H *et al*. (2018) Trisomy of a Down syndrome critical region globally amplifies transcription via HMGN1 overexpression. *Cell Rep* **25**, 1898–1911.
71 Pelleri MC, Cattani C, Vitale L, Antonaros F, Strippoli P, Locatelli C, Cocchi G, Piovesan A & Caracausi M (2018) Integrated quantitative transcriptome maps of human Trisomy 21 tissues and cells. *Front Genet* **9**, 125.
72 Sobol M, Klar J, Laan L, Shahsavani M, Schuster J, Annerén G, Konzer A, Mi J, Bergquist J, Nordlund J *et al*. (2019) Transcriptome and proteome profiling of neural induced pluripotent stem cells from individuals with Down syndrome disclose dynamic dysregulations of key pathways and cellular functions. *Mol Neurobiol* **56**, 7113–7127.
73 Nissanka N & Moraes CT (2018) Mitochondrial DNA damage and reactive oxygen species in neurodegenerative disease. *FEBS Lett* **592**, 728–742.
74 Kimura H (2017) Hydrogen sulfide and polysulfide signaling. *Antioxid Redox Signal* **27**, 619–621.
75 Zhang D, Du J, Tang C, Huang Y & Jin H (2017) H_2S-induced sulfhydration: biological function and detection methodology. *Front Pharmacol* **8**, 608.