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# Review

# Glutathione synthetase deficiency

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**Abstract.** Glutathione (GSH), one of the most important antioxidants in the eukaryotic organism, is synthesized in a two-step procedure where the last step is catalysed by the enzyme glutathione synthetase (GSS). GSS deficiency is inherited autosomal recessively, and patients with this disease can be divided into three groups, according to their clinical phenotype. Mildly affected patients have mutations affecting the stability of the enzyme, causing a compensated haemolytic anaemia; moderately affected patients have, in addition, metabolic acidosis; and severely affected patients also develop neurological defects and show increased susceptibility to bacterial infections. Moderately and severely affected patients have mutations that compromise the catalytic properties of the enzyme. 5-Oxoprolinuria appears in all three groups, but is more pronounced in the two latter groups. Today, no cure can be offered these patients; they are given vitamins C and E to boost their antioxidant levels, and bicarbonate to correct metabolic acidosis.

**Key words.** Glutathione synthetase; 5-oxoprolinuria; metabolic acidosis; haemolytic anaemia; missense mutations; splice mutations; negative cooperativity.

## Glutathione: functional roles and metabolism

An important part of the defence against oxidative stress and free radicals in most organisms is the ubiquitous tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine). It is the most abundant thiol-containing compound in the mammalian cell, with levels as high as 10 mM [1–3]. The highest levels of glutathione are found in liver, spleen, kidney, lens, erythrocytes and leukocytes. Glutathione exists mainly in its reduced form (GSH); only about 1–5% is in oxidized form as glutathione disulphide (GSSG), or as mixed disulphide with small thiol-containing compounds (GSSX) or thiol-containing protein (GSSPr). The NADPH-requiring enzyme glutathione-disulphide reductase reduces GSSG back to GSH, and the GSSG/2GSH ratio is often used as an indicator of the redox status of the cell [4, 5]. Ten to 15% of total intracellular GSH is in the mitochondria [6, 7].

In addition to functioning as an antioxidant, GSH is involved in processes such as regulation of enzyme activities and the synthesis of DNA, proteins and leukotrienes. GSH can detoxify xenobiotics and carcinogens, and it protects against lipid peroxidation and electrophiles [8]. The activity of several eukaryotic transcription factors is regulated by the thiol status of the cell (cf. [9, 10]), and GSH furthermore has an effect on chaperone activity [11]. The tripeptide works as a neurotransmitter [10], neuromodulator [12], and regulator in cell proliferation and apoptosis [13]. The tripeptide also has anti-viral effects [14]. The levels of GSH decrease with age, and low levels of GSH have been observed in numerous diseases, for instance liver cirrhosis, pulmonary disease, diabetes, human immunodeficiency virus (HIV) infection, gastrointestinal and pancreatic inflammation, and neurodegenerative diseases [15, 16].

Although the focus has been on the protective qualities of GSH, it should be remembered that GSH can also give rise to the formation of compounds that are at least as reactive as the parent electrophile itself (cf. [17]).

Haloalkanes and haloalkenes, a widely used group of chemicals, can for instance undergo GSH-dependent bioactivation [18]; the detoxifying qualities of GSH also confer increased resistance to chemo- and radiotherapy (for a review, see [19]); and GSH might also work as an oxidant, generating superoxide anions by transferring electrons from metal ions to molecular oxygen [20].

GSH is metabolised via the γ-glutamyl cycle (fig. 1). The synthesis of GSH is a two-step procedure where the first, and rate-limiting, step is catalysed by glutamate-cysteine ligase (GCL; E.C. 6.3.2.2; formerly γ-glutamylcysteine synthetase). In the reaction, a peptide bond involving the γ-carboxyl group of glutamate is formed. This unusual feature protects the dipeptide from being hydrolysed by intracellular peptidases. GCL is a heterodimer, consisting of one heavy catalytic subunit (GCLC) and one light modulating subunit (GCLM), the latter increasing the affinity of the heavy subunit for its substrate glutamate [21]. The activity of GCL is regulated at multiple levels, allowing a range of stimuli to either induce or inhibit enzyme activity [22].

The enzyme glutathione synthetase (GSS; E.C. 6.3.2.3) adds a glycine to the dipeptide and forms GSH (fig. 2). This enzyme will be described in more detail below. GSH can regulate its own synthesis at physiological concentrations by negative feedback inhibition of GCL, suggesting that the enzyme can respond rapidly to increased need for GSH [23].

The first step in the degradation of GSH is catalysed by γ-glutamyl transferase (GGT; E.C. 2.3.2.2; formerly γ-glutamyltranspeptidase). This ectoenzyme cleaves the γ-glutamyl bond of GSH by transferring the γ-glutamyl residue to an amino acid acceptor. The most active amino acid acceptor is cystine, but other neutral amino acids, especially methionine and glutamine, are also active acceptors. When cystine participates, γ-glutamylcystine is formed by transpeptidation between GSH and cysteine, transported into the cell and reduced by transhydrogenation with GSH to cysteine and γ-glutamylcysteine [24].

The cysteinylglycine part of GSH is hydrolysed into its constituent amino acids by a dipeptidase (E.C. 3.4.13.19). The γ-glutamyl-amino acid is subsequently converted into 5-oxoproline and a free amino acid by γ-glutamyl cyclotransferase (GCT; E.C. 2.3.2.4). 5-Oxoproline is ring-opened by 5-oxoprolinase (5-OPase; E.C. 3.5.2.9), turning 5-oxoproline into glutamate.

At present, deficiencies in humans have been described in four of the six enzymes in the γ-glutamyl cycle: GCL, GSS, GGT and 5-OPase. Although mutations have only been identified in GCL and GSS deficiencies, in all cases the disease seems to be inherited in an autosomal recessive way based on levels of enzyme activity in patients versus parents and controls. For diagnostic purposes it is important to remember that the erythrocytes lack both GGT and 5-OPase.

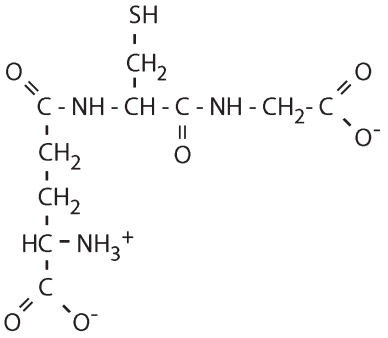


Figure 2. The structure of glutathione. The tripeptide consists of the residues glutamate, cysteine and glycine. Two special features of the structure are the thiol group that gives GSH its reactivity and the peptide bond between the glutamate and cysteine residues, involving the γ-carboxyl group of glutamate instead of the α-carboxyl group normally involved in peptide bonds.

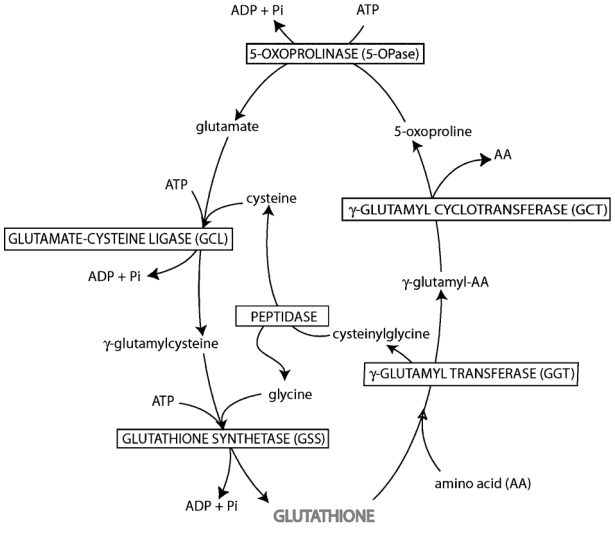


Figure 1. The γ-glutamyl cycle. Six enzymes are involved in the γ-glutamyl cycle (boxes), two on the synthetic side and four on the degradative side.

## GSS deficiency

About 70 patients have been described worldwide with GSS deficiency (MIM 266130) [25]. Approximately 25% of GSS-deficient patients die during the neonatal period of infection and electrolyte imbalance. The patients can be divided into three groups according to their clinical manifestation [26]. The mild form is most likely caused by mutations affecting the stability of the enzyme [27]. Nucleated cells have a high enough protein turnover to compensate for this stability defect. However, as GSH is essential for cell membrane integrity, the lack of protein synthesis in mature erythrocytes results in isolated haemolytic anaemia.

Moderately affected patients develop metabolic acidosis and a massive excretion of 5-oxoproline in the urine (0.5–1 g/kg bw/day). In a healthy person, synthesis of GSH is favoured because GSS has a higher affinity than GCT for the dipeptide [28]. In GSS-deficient patients, however, γ-glutamylcysteine accumulates because of the block in the downstream step of the cycle and decreased negative feedback inhibition of GCL [29]. γ-Glutamylcysteine is hydrolysed back into 5-oxoproline and cysteine by GCT. The buildup of 5-oxoproline exceeds the capacity of 5-OPase, 5-oxoproline (also known as pyroglutamic acid) levels rise in body fluids and the metabolite is excreted via the urine. The GSS deficiency therefore gives rise to a modified γ-glutamyl cycle, lacking GSS and omitting the step catalysed by the dipeptidase.

In addition to the features above, severely affected patients develop, progressive dysfunction of the central nervous system (CNS), including motor functional disturbances and mental retardation. They show an increased susceptibility to bacterial infections, probably caused by defective granulocyte function, and in some cases pathological electroretinograms and retinal pigmentation [30]. The disease has also been associated with antenatal cerebral haemorrhage [31], low levels of cysteinyl leukotrienes [32, 33] and multiple malformations [34].

γ-Glutamylcysteine accumulates in fibroblasts of GSS-deficient patients, and it has been suggested that the dipeptide may substitute for some of the functions of the tripeptide [29]. In yeast, γ-glutamylcysteine protects just as well as GSH against oxidative stress, but cannot fully substitute for other essential functions of GSH implied by poor growth of a GSS-deficient mutant yeast strain on a minimal medium [35].

One pregnancy has been reported in a moderately affected GSS-deficient patient, resulting in a healthy baby, born after 41 weeks of gestation [36]. The levels of 5-oxoprolinuria and metabolic acidosis in the mother remained stable during the pregnancy, and no negative effects of the pregnancy on the GSS deficiency could be detected. As a neonate, the child had mild transient jaundice that resolved without treatment; at 4 years of age the child was healthy, and psychomotor development was normal.

## Pathology in GSS deficiency

Autopsy has been performed in only a few patients with GSS deficiency. The first GSS-deficient patient diagnosed died at 28 years of age [37]. Examination of the brain showed focal lesions in the frontoparietal cortex, bilateral focal lesions in the visual cortex and thalamus, and selective atrophy of the granule cell layer of the cerebellum [38]. Other GSS-deficient patients who died during the neonatal period showed signs of cerebral necrosis, intrauterine hypoxia, and generalized cerebral atrophy with asymmetric and partly enlarged gyri, infection and haemorrhage. Thus far, no consistent neruopathological pattern has been revealed.

## Diagnostics

Most patients with GSS deficiency present with severe metabolic acidosis (without ketosis or hypoglycemia), haemolytic anaemia and jaundice during the perinatal period. High levels of 5-oxoproline in the urine and low GSS activity in erythrocytes or fibroblasts (approximately 10% of control values) indicate GSS deficiency. Heterozygous carriers have an enzyme activity of 50–70% of control values. Mutation analyses establish the diagnosis. Because of the high percentage of splice mutations (approximately 40%), we recommend that mutation analyses at the genomic level should be complemented with analyses of RNA transcripts [39]. Interestingly, patients with splice mutations and undetectable levels of protein using a polyclonal GSS-specific antibody show residual enzyme activity, albeit very low [39]. The mechanism behind this phenomenon remains to be established. Prenatal diagnosis can be performed by analysis of GSS activity in cultured amniocytes [40], or chorion villi samples [unpublished results] or by measuring the levels of 5-oxoproline in amniotic fluid [41].

5-Oxoprolinuria may have other aetiology than defects in the GSS enzyme, and these should be considered in diagnostic work [42]. To a varying extent, 5-oxoprolinuria has, for instance, been found associated with:

- 5-Oxoprolinase deficiency (described below).
- Some infant formulas containing casein, which can be converted to 5-oxoproline upon heating [43].
- Chemical treatment of tomatoes during production of tomato juice, which turns glutamine into 5-oxoproline [44].
- Severe burns, where decreased GSH synthesis is probably caused by lowered availability of glycine [45–47]. It has been suggested that 5-oxoprolinuria can be used as an indicator of glycine insufficiency [48].
- Stevens-Johnson syndrome [42], where patients have an increased production of proteins containing N-terminal 5-oxoproline, such as fibrinogen and collagen.
- Homocystinuria. 5-Oxoprolinuria was reported in one patient where the plasma level of homocystine was above 0.2 mM [49]. GCL may use homocysteine as a substrate and form γ-glutamylhomocysteine, which can be split by GCT into 5-oxoproline and homocysteine.
- Defects of the urea cycle [50]. The high levels of 5-oxoproline in the urine of these patients were seen in association with increased levels of total amino acid excretion. Intraperitoneal administration of a mixture of amino acids to mice cause an increase in the 5-oxoproline levels, especially in the kidney, possibly by inducing GGT [51].

- Prematurity: transient 5-oxoprolinuria can be seen in preterm infants, probably related to insufficient supply of glycine [52].
- Administration of some drugs, such as vigabatrin [53], and paracetamol [54]. When feeding rats with paracetamol, only 25–30% of the paracetamol was metabolised with GSH to the cysteine conjugate [55]. Consequently, an additional step in the γ-glutamyl cycle was suggested to metabolise the bulk of 5-oxoproline, forming GSH by direct conjugation of 5-oxoproline and cysteine. In the case of cysteine insufficiency (due to paracetamol conjugation or low protein diet), there would be an overload of 5-oxoproline secondary to antiproliferative and antimicrobial drug treatment [56].

## Genotype-phenotype correlation

As the knowledge of disease-causing mutations in the human genome has increased, much effort has been directed at correlating genotype and phenotype in genetic diseases. If molecular diagnosis could predict the phenotype, this would increase the benefits of early postnatal as well as prenatal diagnosis. A study of the correlation between genotype and phenotype in 41 patients with GSS deficiency was performed [57]. Even though the differences among the clinical phenotypes with respect to enzyme activity, GSH or γ-glutamylcysteine in cultured fibroblasts did not reach statistical significance, the clinical phenotype could to some extent be predicted based on the type of mutation involved. All mutations causing aberrant splicing, frameshift, or premature stop codons were associated with the moderate or severe clinical phenotypes. In addition, residual GSS activity correlated with GSH levels in cultured fibroblasts. The data indicate that the phenotype, especially of the two more severe clinical forms, is modified by additional genetic or environmental factors. Also, the phenotype classification is not distinct as patients may develop neurological or retinal pathology over time [unpublished observations].

## Treatment

Patients with GSS deficiency are given sodium bicarbonate, citrate or trometamol (THAM) to correct the metabolic acidosis. To boost the levels of free radical scavengers, patients receive high doses of vitamin C (100 mg/kg body weight/day) and vitamin E (10 mg/kg body weight/day). The latter is also given to correct granulocyte dysfunction [58, 59]. In the correlation study between phenotype and genotype in 41 GSS-deficient patients, only 1 of the 18 severely affected patients had received both vitamins C and E from the neonatal period, in contrast to 6 of the 17 moderately affected patients, suggesting that early onset of treatment with both vitamins may be beneficial [57]. Previously, some patients received N-acetylcysteine (NAC) as a supplement (15 mg/kg body weight/day). However, this is no longer recommended, as cultured fibroblasts from GSS-deficient patients show increased levels of cysteine [29], which is neurotoxic in excessive amounts [60], and NAC may increase the levels of cysteine even further. Drugs that are known to cause haemolytic crises in patients with glucose-6-phosphate dehydrogenase deficiency (G6PD), e.g. sulphonamides and phenobarbitals, should be avoided.

Administration of several cysteine delivery compounds, such as NAC, 2-oxothiazolidine-4-carboxylate (OTC; a 5-oxoproline analogue), methionine and S,N-diacetylcysteine monoethyl ester, increase the intracellular levels of GSH [61, 62]. However, these compounds would generally be of limited value in raising GSH levels in GSS-deficient patients, as these patients are unable to form the tripeptide. Administration of γ-glutamylcyst(e)ine would increase intracellular GSH levels [61] and bypass the GSH-regulated step catalysed by GCL, but the action of GSS is nevertheless needed to form GSH.

GSH administered orally is hydrolysed by dipeptidase in the gastrointestinal tract [63], and following intravenous administration GSH has a half-life of only 2 min. As administered GSH is broken down extracellularly, it will function as a cysteine delivery compound. In contrast to the widely held view that GSH does not readily cross the blood-brain barrier [64, 65], GSH transporters have been reported in human brain endothelial cells and human astrocytes [66]. (For a review of GSH pathways in the brain, see [67].) The significance of these transporters in GSH homeostasis of the human brain remains to be established.

Glutathione mono- and diesters have been investigated as GSH-delivery compounds [61]. They are lipid soluble and readily cross the cell membrane before they are hydrolysed into GSH and the corresponding alcohol, usually ethanol. None seems to hold great promise for future clinical trial. However, the GSH-derivative S-acetyl-GSH shows promising results, as exposure of cultured fibroblasts from GSS-deficient patients to this compound restored intracellular GSH levels to within normal range [68].

A potential approach for treating GSS-deficient patients might be enzyme replacement therapy. Modifying enzymes with polyethylene glycol (PEG) can prolong the circulating life of enzymes and lower their immunogenicity. PEG-modified adenosine deaminase (PEG-ADA) has been used for almost 20 years in the treatment of patients with ADA deficiency and has also allowed gene therapy in this disease [69]. Superoxide dismutase and catalase modified with PEG prolong the survival time of rats under hyperoxia [70].

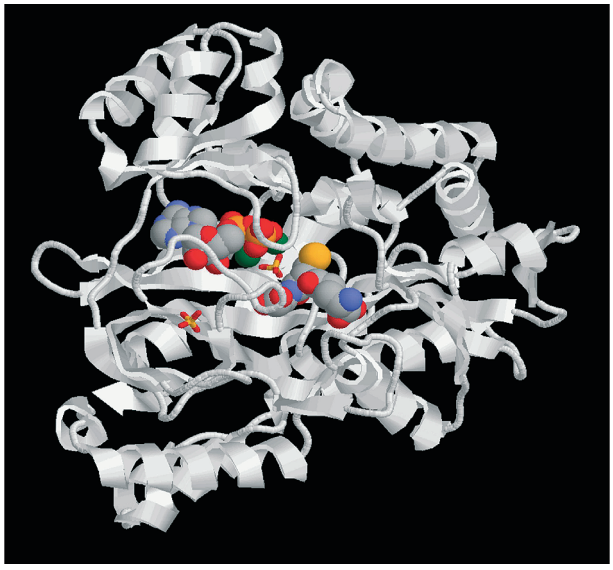

Figure 3. A molecular model of the human GSS monomer. GSH and ADP are shown as space-filling figures (with carbon coloured grey, oxygen red, nitrogen blue, phosphorous orange and sulphur yellow). Two magnesium ions essential for the reaction are shown as green spheres and two sulphate anions are shown as ball-and-stick figures. The figure is based on the crystal structure of human GSS (Protein Data Bank; http://www.rcsb.org/pdb, code 2HGS [74]) and was generated using Protein Explorer [92].

## Molecular aspects of GSS

The human GSS enzyme is encoded by a single-copy gene on chromosome 20q11.2 [71] containing 13 exons, 12 coding and one non-coding upstream of the transcriptional start site [72, 73]. The enzyme functions as a homodimer where each subunit contains 474 amino acid residues and has a molecular mass of 52 kDa. The three-dimensional structure of human GSS is known [74] (fig. 3), and the enzyme has been identified as a member of the ATP-grasp superfamily [74], where a common feature is carboxylate-amine/thiol ligase activity [75]. GSS is conserved through evolution with an amino acid identity of 89% with *Mus musculus*, 65% with *Xenopus laevis* and 36% with *Schizosaccharomyces pombe* [72]. In contrast to this, the *Escherichia coli* enzyme is a tetramer, with subunits of only 316 amino acids [76] and with no obvious sequence similarity to the human enzyme.

Mutation analyses in GSS deficiency have revealed mostly missense mutations (fig. 4); however, one-third of the mutations described so far (comprising >40% of the analysed alleles, siblings counted only once) are splice mutations [57]. These mutations cause exon skipping, pseudoexons, partial exclusion of exonic sequences and retention of downstream intronic sequences [39]. In total, 16 missense mutations, 9 splice mutations, 2 deletions, one insertion and one nonsense mutation have been identified in GSS deficiency [25].

### Kinetics of GSS

Whenever there is more than one binding site for a substrate in an enzyme, there is a possibility for cooperativity between the sites. Kinetic analyses of human recombinant GSS showed that binding of its γ-glutamyl dipeptide substrate does not follow conventional Michaelis-Menten kinetics, but rather displays negative cooperativity [77]. Cooperativity, both negative and positive, is often observed in enzymes at crucial steps in metabolism. However, the physiological consequence of the negative cooperativity displayed by GSS towards the γ-glutamyl substrate is not clear. GSH is involved in numerous processes in the cell and regulates its own synthesis by negative feedback inhibition of GCL. Local fluctuations in the levels of GSH might therefore cause oscillations in the level of γ-glutamylcysteine. Since negative cooperativity supposedly makes enzymes less sensitive to changes in substrate concentrations, the allostery would counteract such fluctuations and emphasizes the importance of keeping GSH production constant.

Kinetic analyses of recombinant human GSS containing naturally occurring missense mutations showed that these affect the stability, catalytic capacity, substrate affinity and cooperativity of the enzyme [78, 79].

## Other inborn errors of the γ-glutamyl cycle

### γ-GCT deficiency

Five patients have so far been reported with GGT deficiency (cf. [25]). In addition to low activity of enzyme, patients exhibited glutathionaemia, glutathionuria (up to 1 g/day; controls <10 mg/day), and increased levels of γ-glutamylcysteine and cysteine in the urine. Three patients had mental retardation and symptoms of degeneration of the CNS. However, as these patients were identified when screening mentally retarded patients, the linkage of the CNS impairments to the enzyme deficiency remains to be established [80, 81]. The diagnosis is based on the finding of increased levels of GSH in urine and plasma and lowered enzyme activity levels.

A mouse model for GGT deficiency (*Ggt1*$^{-/-}$) appears normal, but the mice grow more slowly than wild-type mice. They are sexually immature, develop cataracts, and most die between 10 and 18 weeks of age [82].

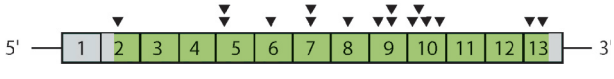


Figure 4. The distribution of the 16 missense mutations identified in GSS deficiency. The figure illustrates the cDNA (complementary DNA), grey areas are nontranslated regions, exons are numbered.

### 5-Oxoprolinase deficiency

Eight patients with 5-oxoprolinase deficiency (MIM 260005) have been reported (cf. [25]). The reduced conversion of 5-oxoproline to glutamate causes 5-oxoprolinuria (0.1–0.5 g/kg bw/day), though not to the same extent as in GSS-deficient patients, and usually not in combination with metabolic acidosis. In some patients mental retardation, microcephaly, microcytic anaemia, hypoglycaemia, renal stones and enterocolitis have been reported. The diagnosis is based on the finding of 5-oxoprolinuria and decreased enzyme activity.

### GCL deficiency

GCL deficiency (MIM 230450) has been reported in eight patients, including one sibling pair (cf. [25]). Mild haemolytic anaemia is a common clinical finding in these patients. One patient had learning disability with dyslexia and was regarded as mentally retarded [83], and the sibling pair had generalised aminoaciduria, spinocerebellar degeneration and later on developed a neuromuscular disorder with peripheral neuropathy and myopathy [84]. The sister in this sibling pair became psychotic after treatment with sulphonamide for a urinary tract infection. The diagnosis of this deficiency is based on the low levels of γ-glutamylcysteine and GSH in erythrocytes together with low enzyme activity. Mutation analyses have been performed in five of the patients, revealing three different missense mutations in the heavy subunit of the heterodimer [83, 85, 86]. Several polymorphisms in the 5′-flanking region of both involved genes, *Gclc* and *Gclm,* has been identified, and individuals heterozygous for these single-nucleotide polymorphisms show increased risk of myocardial infarction [87, 88].

Mice homozygous for a disruption in the *Gclc* gene (*Gclc*$^{-/-}$) fail to gastrulate beyond embryonic day 8.5 (E8.5; day of vaginal plug defined as E0.5) and have a high rate of distal apoptosis [89, 90]. Even though the embryos could not be rescued using NAC [89] or GSH ethyl ester [90], blastocysts from the embryos could be cultured if NAC or GSH was added.

Mice homozygous for a disruption in the *Gclm* gene (*Gclm*$^{-/-}$) lack an overt phenotype; however, the female *Gclm*$^{-/-}$ mice gain weight slightly more slowly than both the female control animals and the heterozygous carrier mice [91]. Interestingly, GSH levels in liver, kidney, pancreas, erythrocytes and plasma of this knockout mouse are lower than in GSS-deficient patients [29], and haemolytic anaemia is absent.

### Conclusion

The evidence supporting the importance of GSH homeostasis during, for instance, oxidative stress is immense, and the (patho)physiological role of GSH is under intense investigation as evidenced by the rapidly increasing number of publications on this tripeptide, constantly improving and changing our knowledge. A method to increase the intracellular GSH levels, especially in the brain, would be optimal for GSS-deficient patients. As low levels of GSH are seen in a substantial number of clinical conditions, any such method would also undoubtedly be of interest for other patient groups.

Several mouse models with low GSH levels exist. These will hopefully provide more information about the role of GSH in different processes, during embryogenesis, adult life and senescence. In addition, they will be valuable in developing methods to increase the intracellular levels of this all-important compound.

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