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Review

Impact of CHO Metabolism on Cell Growth and Protein Production: An Overview of Toxic and Inhibiting Metabolites and Nutrients†

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Abbreviations: **ADP**, adenosine monophosphate; **AMP**, adenosine monophosphate; **Anpep**, alanyl aminopeptidase membrane; **ATP**, Adenosine triphosphate; **BAK**, Bcl-2 antagonist killer protein; **BAX**, Bcl-2-associated X protein; **Cas** , CRISPR-associated; **Cas9**, Cas protein 9; **CHO**, Chinese hamster ovary; **CRISPR**, Clustered regularly interspaced short palindromic repeats; **EPO**, erythropoietin; **ER**, Endoplasmatic Reticulum; **FUT8**, fucosyltransferase 8; **Gcl**, Glutamamte cysteine ligase; **Gclc**, Glutamamte cysteine ligase catalytic subunit; **Gclm**, Glutamamte cysteine ligase regulatory subunit; **GDP**, guanosine diphosphate **GEM(s),** genome-scale models; **Ggt**, gamma-glutamyltransferase; **Ggct**, gamma-glutamylcyclotransferase; **GMP**, guanosine monophosphate; **Gpx**, glutathione peroxidase; **GS**, glutamine synthetase, **Gsr**, Glutathione-disulfide reductase; **GSH**, reduced glutathione; **Gss**, Glutathione synthetase; **Gst**, glutathione *S*-transferase; **GSSG**, oxidized glutathione or glutathione disulfide; **G3P**, glycerol-3-phosphate; **G3PC**, glycero-3-phosphocholine; **G6d**, glucose-6-phosphate**; G6pd**, glucose-6-phosphate dehydrogenase**;** **HMDB**, Human Metabolome Database; **KEGG**, Kyoto Encyclopedia of Genes and Genome; **LdhA**, lactate dehydrogenase A; **mAb(s)**, monoclonal antibody(ies); **MAS**, malate–aspartate shuttle (MAS); **MDHII,** malate dehydrogenase II; **NaCl**, sodium chloride; **NAD^+**, Nicotinamide adenine dinucleotide oxidized; **NADH,** Nicotinamide adenine dinucleotide reduced; **$NADP^+$**, Nicotinamide adenine dinucleotide phosphate oxidized; **NADPH**, Nicotinamide adenine dinucleotide phosphate reduced; **PCHO**, Choline phosphate; **Pdhk**, pyruvate dehydrogenase kinase; **PYC2**, yeast pyruvate carboxylase **TALENs**, transcription activator-like effector nucleases; **TCA**, Tricarboxylic acid; **tPA**, tissue plasminogen activator; **Txndc12**, thioredoxin domain containing 12; **ZFNs**, zinc finger nucleases.

Abstract

For over three decades, Chinese hamster ovary (CHO) cells have been the chosen expression platform for the production of therapeutic proteins with complex post-translational modifications. However, the metabolism of these cells is far from perfect and optimized, and requires substantial knowhow and process optimization and monitoring to perform efficiently. One of the main reasons for this is the production and accumulation of toxic and growth-inhibiting metabolites during culture. Lactate and ammonium are the most known, but many more have been identified. In this review, we present an overview of metabolites that deplete and accumulate throughout the course of cultivations with toxic and growth inhibitory effects to the cells. We further provide an overview of the CHO metabolism with emphasis to metabolic pathways of amino acids, glutathione (GSH), and related compounds which have growth-inhibiting and/or toxic effect on the cells. Additionally, we survey relevant publications which describe the applications of metabolomics as a powerful tool for revealing which reactions occur in the cell under certain conditions and identify growth-inhibiting and toxic metabolite. We also present a number of resources that describe the cellular mechanisms of CHO and are available on-line. Finally, we discuss the application of this knowledge for bioprocess and medium development and cell line engineering.

1. Introduction

Chinese Hamster Ovary (CHO) cells are the mammalian host of choice for the production of recombinant biological compounds. The market of therapeutic recombinant proteins presents cumulative sales values, ranging between $107 to $140 billion from 2010 to 2013 [1]. The first drug produced in this expression system was tissue plasminogen activator (tPA), which reached the marked in 1987 [1]. Examples of products expressed in CHO cells include erythropoietin (EPO) indicated for the treatment of severe anemia, coagulation factors as factor IX used as a therapeutic in hemophilia, interferon used for treating multiple sclerosis and monoclonal antibodies (mAbs) with the indication for treating Crohn's disease, different lymphomas, and cancers (e.g., breast and gastric cancer) [1]. From the biological drugs approved between 2006 and 2010, about 55% were produced in mammalian cells [2, 3]: from those between 2010 to the middle of 2014, 60% of the recombinant therapeutic proteins were also produced in mammalian cells. This shows an increasing trend which favors the use of expression systems of mammalian origin. Under the latter-mentioned time period, 33% of total approvals were for drugs expressed in CHO cells [1]. In retrospect, CHO cells have shown to be safe hosts and therefore, more likely to obtain approval for novel therapeutic proteins manufactured in this cell platform by the regulatory agencies.

The main advantages of using CHO cells compared to other microbial or mammalian cells [4] include the ability of these cells to perform post-translational modifications similar to those found in human proteins, such as glycosylation, which is considered to be a critical quality attribute. The presence of an aberrant glycan profile will decrease the efficacy [5], affects the protein drug pharmacokinetics [6], and alters biological properties [7–10]. In addition, CHO cells have been demonstrated to display reduced susceptibility to human viral infections [11], which represents an additional advantage over cell lines of human

origin. Genomic and transcriptomic analysis of CHO-K1 showed that genes encoding for viral entry receptors, as well as other genes required for a successful viral infection, are absent or not expressed in the cell line [12]. CHO cells can grow in chemically defined medium, which reduces the chances for batch-to-batch variation and have the ability to be cultured in suspension, to facilitate the scale-up of the bioprocess [13].

The metabolism of CHO cells, characterized by high uptake rates of substrates used as carbon and nitrogen sources [14–16], is generally inefficient and suboptimal. The nutrients supplied in the media and feeds at certain concentrations may lead to the accumulation of metabolites, intermediates, and by-products. This indicates the existence of metabolic bottlenecks in key pathways and inefficient flux distribution. Furthermore, these accumulating compounds may decrease cell growth, productivity [17, 18], and protein quality [19, 20]. As monitoring the metabolites and changing the related pathways has the potential to improve recombinant protein production in CHO cell culture, these 45 compounds are presented in a tabulated form (Table 1). The table contains the main reports on the effects of the individual metabolites, and provides helpful primary references on e.g. the effect of concentrations of individual amino acids and sugars.

Furthermore, for some of these compounds, the reported effects are complex and surprising. To provide additional detail and context of these metabolites, we present overviews of the pathways for generation and consumption of the main toxic and inhibiting metabolites; glycolysis, the tricarboxylic acid (TCA) cycle, and amino acid metabolism, as well as glutathione metabolism. Moreover, some lipids have been shown to affect growth, for which we also discuss the details. In addition, we present methods and methodologies, which can be used to decrease or remove the presence of such toxic and inhibiting metabolites.

2. An Overview of CHO Metabolism

CHO cells have an inefficient metabolism, which is characterized by high uptake rates of substrates used as carbon and nitrogen sources (e.g., glucose and glutamine [14]). The substrates are not fully used for production of biomass or recombinant proteins: thus, based on reports, 35% [21] and 70% [22] of glucose can be diverted into the formation of waste products, which impact the cell culture performance [15, 21, 22]. Examples of toxic or inhibiting metabolites can be found throughout metabolism [17–19]. Table 1 summarizes compounds that are reported to correlate with cell growth inhibition, apoptosis and/or have additional negative effect in culture due to accumulation or depletion. These metabolites are reporters of metabolic inefficiency and represent a waste of carbon diverting from the main metabolic pathways. Additionally, some of the listed metabolites function as alternative redox sinks (sorbitol, threitol, and glycerol), while others (amino acids) are catabolized instead of contributing directly to recombinant protein production and lead to the formation of toxic intermediates. In this review, we have chosen to focus on the main pathways where the majority of these compounds have been reported; glycolysis, the TCA cycle, amino acid metabolism, and glutathione (GSH) metabolism.

2.1. Central metabolism: nutrient uptake and by-product formation

The main carbon source in CHO cells is glucose, which is supplied in media and feeds that are used in batch and fed-batch bioprocesses. Glucose is taken up by the cell at high rates and phosphorylated to glucose-6-phosphate (G6P), and used in glycolysis to form adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH) and pyruvate. Instead of proceeding to the full oxidation of glucose in aerobic conditions, pyruvate is converted into lactate along with the oxidation of NADH to oxidized nicotinamide adenine dinucleotide (NAD^+) by the action of lactate dehydrogenase A (LdhA). This represents a diversion of a flux of carbon away from the TCA cycle, to lessen the energy production and decrease production of important $C_{4\text{-}6}$ precursors which are

required for biomass formation (Figure 1). This phenomenon is also observed in cancer cells and called the Warburg effect [23]. For a better understanding of the relation between NAD^+/NADH and glycolysis, we suggest reading the review by J. Locasale and L. Canteley [24].

As seen in Figure 1, lactate is one of the main toxic metabolites found in central metabolism. The consequences of the accumulation of lactate in mammalian cell culture have been widely mentioned in the literature (Table 1). Reports have shown that lactate inhibits cell growth, induces apoptosis and reduces productivity of recombinant therapeutic products, due to changes in pH and osmolality [17, 18, 25–27]. Many other studies have explored the underlying motives for such phenotype [28–34]. In a bioprocess, two distinct phases of lactate metabolism have been described; initially, glucose consumption is accompanied by lactate production whilst, in later phases, the consumption of lactate is observed, although simultaneous consumption of glucose and lactate has likewise been described [29, 34]. It is important to note the link between lactate consumption phenotype and increased productivity [35], as well as the metabolic shift from lactate production to lactate consumption as a marker of metabolic efficiency [33]. Reports show that initial lactate supplementation can induce a shift from high to low glycolytic flux, even in the presence of high glucose concentration [36]. When lactate is added into the medium along with pyruvate, glucose uptake rate was reduced by 50% [37]. For additional detail, the role of lactate has been extensively reviewed. We suggest the reader examines a set of particularly excellent reviews and research papers [15, 16, 28, 38].

Alternative fates for carbon have also been suggested since glucose can also be converted into glycerol along with oxidation of NADH to NAD^+, as well as sorbitol and threitol – in both cases, accompanied by the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$).

These compounds are formed from glycolytic intermediates and accumulate both intracellularly and extracellularly in the transition of the exponential to stationary phase of culture, after the addition of feed containing depleted nutrients (Table 1) [26, 39]. Additionally, G6P is shunted away from glycolysis to enter the pentose phosphate pathway (PPP) where the sugar precursors are required for the synthesis of nucleotides, NADPH, and glycolytic intermediates are produced. The presence of nucleosides and nucleotides – adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), guanosine diphosphate (GDP), and guanosine monophosphate (GMP) – in the culture medium at low concentrations (1 mM) has shown to arrest cell growth and to contribute to protein production [40]. However, as specified in Table 1, adenosine, ADP, and AMP have been reported to be cytotoxic. In particular, the extracellular concentrations of AMP as high as 2 mM become cytotoxic [40] while both AMP and GMP have been correlated with early apoptotic events in CHO cells [41].

An oxidative metabolism is characterized by the channeling of the carbon molecules from glycolysis into the TCA cycle. Intermediates of the TCA cycle (citrate, succinate, fumarate and malate) accumulate during culture phases, which indicates a bottleneck (Table 1) (reviewed by Dickson [42]). These intermediates were observed to build up in the cell culture medium after the addition of a feed, which contains pyruvate and amino acids (aspartate, asparagine and glutamate), and has been linked to growth limitation [26, 39, 43].

2.2. Amino Acid Metabolism in CHO Cell Culture

In CHO cell culture, amino acids are supplied in the growth medium and/or produced via biosynthetic pathways. These are required to support cellular functions, such as cell growth, and utilized as building blocks for protein synthesis [44, 45]. Through the catabolism of amino acids, the cell can utilize the carbon backbones for the formation of TCA cycle intermediates, which are to be used in the central metabolic pathways (Figure

1). When these are supplied in excess, a wasteful cellular metabolism will lead to the formation of by-products, in particular ammonium. Ammonium is mainly formed from the breakdown of glutamine. The catabolism of several amino acids also leads to the formation of ammonium. This occurs via transamination reaction as an amino group is transferred to α-ketoglutarate and forming glutamate – that, in its turn, is deaminated to release ammonium ion, $NH4^+$ [46]. Amino acids, such as Serine and Threonine, can undergo direct deamination. [46]. Ammonia has a negative impact on the product quality attributes when it accumulates and, similarly, affects productivity and cell growth [18–20, 25, 47, 48]. The mechanism by which ammonia affects growth is still not fully understood. It has been reported that the increasing concentration of ammonia modifies the electrochemical gradient and acidifies the intracellular milieu, which disrupts enzymatic activity and leads to apoptosis [18, 49]. Cell growth is inhibited in mammalian cell lines by ammonia concentrations ranging from 1.8 to 33 mM [50]. For CHO cells, cell growth inhibition has been reported for an ammonia concentration of 5.1 mM [25] and a reduction of 50% of growth was observed for ammonia concentrations were above 8 mM [51]. In an additional report, apoptotic cell death was not detected when CHO cells were exposed to 50 mM of ammonium chloride upon engineering of apoptotic genes [52]. Therefore, in a chemically-defined medium, the initial amino acid concentrations should be well controlled and adjusted to the cell's specific metabolic requirements, based on prior studies of the cell line [50].

2.2.1. Amino Acid Catabolism Leads to Formation of Toxic Intermediates

Changes in amino acid concentrations at defined culture phases have been correlated to cell growth inhibition and cell death. For example, asparagine consumption [26, 39, 53] has a negative effect in cell growth, [53, 54], while alanine production [26, 39, 53] inhibits TCA cycle [53, 54] and also represents a source of ammonium [55]. In another study, lysine was supplied in excess in media formulations (considering a relatively low cell

density) and accumulates during the death phase [56]. A more complete description is available in Table 1. Interestingly, it has recently been shown that the catabolism of phenylalanine, tyrosine, tryptophan, methionine, leucine, serine, threonine, and glycine leads to the formation of nine intermediates (Table 1), which are identified to be inhibiting cell growth [57, 58]. These metabolites should, in principle, be immediately converted to the next metabolite in the catabolic pathway and, thus, end up in the TCA cycle. However, the pathways are not optimally regulated and, thus, under conditions of low lactate and ammonia, and high cell density observed in the later stages of cultivation, "leak" the inhibiting compounds into the medium, where it accumulates. Furthermore, it was demonstrated that controlling the concentrations of these amino acids resulted in a reduction of the formation of inhibitory intermediates and improved cell growth and product titers during fed-batch cultivation for antibody production [57].

2.3. Control of Lipid Metabolism is Required for Productive Cell Culture

A number of lipids have been shown to deplete or build up over time in CHO cell culture (Table 1). In particular, glycerolipids have been seen in multiple studies to affect growth in a variety of ways [26, 39, 42, 43, 59]. Choline phosphate (PCHO), glycero-3-phosphocholine (G3PC), and glycerol-3-phosphate (G3P) have all been shown to build up in culture over time, which has been seen to lead to growth limitation in both fed-batch and batch cultures [26, 42, 43, 59]. This is an interesting phenomenon, as it suggests a poor regulation of glycerolipids and membrane composition in CHO cells.

PCHO has also been reported to deplete over time in longer cultivation, which also leads to growth limitations, possibly due to a resulting build up of G3P in the culture [59]. Finally, one can also see glycerol as linked to glycerolipids, despite the compound having many other functions in the cell, such as an osmotic regulator and a storage compound/redox sink [39]. As such, this makes the monitoring of glycerol over culture interesting, as it has been reported to also accumulate in the cells over culture [26] [43]. Given the many roles

of glycerol, it is difficult to evaluate the reason or effect of this accumulation, but the ties with the redox potential of the cell and the possible links to lactate metabolism through NADH metabolism makes it an intriguing prospect.

Overall, the metabolism of glycerol and glycerolipids appear to be suboptimal in CHO cells and with possible growth-limiting or -arresting effects. It is thus clear that these aspects must be tightly controlled for optimal cell culture performance.

2.4. Glutathione Metabolism

Glutathione is a small abundant non-protein thiol, which is assembled from three amino acids found in eukaryotic cells [60, 61]. In mammals, its main role is to act as a protective molecule against oxidative stress by transitioning between the oxidized (GSSG) and reduced (GSH) form of the molecule (Figure 2), and have as such been linked to cellular stress responses. In particular, glutathione participates in redox signaling, detoxification of xenobiotics, regulation of cell proliferation, apoptosis, and is involved in immune function events [60, 62]. While GSH and GSSG are important for the overall cellular metabolism, we focused on the key mechanism regarding recombinant protein production in this review.

2.4.1. Biosynthesis of Glutathione

The *de novo* biosynthesis of GSH takes place in the cytosol, as the first reaction is catalyzed by the enzyme glutamate-cysteine ligase (Gcl) which assembles the amino acids cysteine and glutamate at the cost of one ATP to form γ-glutamyl-cysteine – this represents the rate-limiting step in this pathway. Gcl is an enzyme composed of two subunits that are coded by two different gene sequences as seen in higher eukaryotes – these subunits are the catalytic (Gclc) and the modifier (Gclm). Gcl activity is regulated by the concentration of GSH present in the cell in a feedback inhibition manner [63]. Furthermore, the availability of cysteine, as it donates the sulphur from its sidechain, represents another important limiting factor in the biosynthesis of GSH. The second step in the generation of

GSH is catalysed by the enzyme glutathione synthase (Gss) where glycine is added to γ-glutamyl-cysteine, also at the cost of one ATP to form γ-L-glutamyl-L-cysteinyl-glycine – GSH (Figure 2).

As GSH interacts with reactive oxygen species and other redox proteins, it is converted into glutathione disulphide (GSSG), where two molecules of GSH are required to form one GSSG. The salvage pathway of glutathione formation occurs when GSSG is reduced back to GSH in a reaction catalyzed by glutathione-disulfide reductase (Gsr) which requires the availability of NADPH as a co-factor, along with the magnesium ion Mg^{2+}. Post-translational regulation of Gcl involves modifications of Gclc via phosphorylation, caspase-mediated cleavage, which may have a mild impact on overall Gcl activity [64] . Additionally, $NADP^{+}$ and NADPH can also modulate Gcl activity *in vitro* [64]. Glutathione, additionally, can detoxify the cell from the toxic compound methylglyoxal (Table 1), which is formed spontaneously as its free form reacts with GSH [65].

2.4.2. Glutathione in the Context of Recombinant Protein Production

During the production of recombinant proteins, the cell metabolism is characterized by high glycolytic metabolism during cell growth, while maximum antibody production is associated with a more oxidative metabolism [21]. Due to their high proliferative nature, CHO cells may experience increased levels of oxidative stress and, consequently, require higher levels of GSH [66, 67], as observed in most types of cancer cells. Chong et al. [41] used a metabolomics approach with LC-MS analysis for the identification of compounds which induced apoptosis in a fed-batch cultivation of a mAb-producing CHO cell line. The shortlisted extracellular metabolites which correlated with intracellular caspase activity were GSSG, AMP, GMP, and amino acid derivatives, which included dimethylarginine and acetylphenylalanine (Table 1). In particular, the presence of GSSG in the medium resulted in an increased fold-change in caspase activity, which showed a strong link between GSSG accumulation and the early signal of apoptotic cell death. This observation suggests that

GSSG is an additional cause for cell death in prolonged cell cultures, other than those linked to lactate and ammonia. In a subsequent study, the group defined the GSH as a marker of productivity, as high mAb producers have high intracellular GSH content [68]. This same trait was observed in CHO cells that produced a different mAb in a study which combined different proteomics methods to determine differentially expressed proteins [69]. Interestingly, this study has also shown that, amongst other pathways, glutathione biosynthesis enzymes were upregulated in the producer cells. The engineering strategy developed by the same group is further discussed in section 4.

3. Metabolomics as an Evaluator of Presence of Growth-inhibiting and Toxic Metabolites

Metabolomics allows the quantitative analysis of metabolites that are present inside and outside the cell, and provides evidence regarding which pathways and reactions are active in the cell under given culture conditions [70–72]. Metabolomics represents a complement to other 'omics [73], since the data gathered from these investigations can also be integrated into metabolic models. While metabolic profiling is employed when a small set of metabolites that are linked to a phenotype is known, metabolomics is employed to measure and identify all possible metabolites and, consequently, explore hitherto undetermined metabolic links to a phenotype [34]. Thus the cell metabolome, along with the other 'omics data, can inform which cellular events are responsible for a specific phenotype (e.g., high protein producer). When reviewing metabolomics in the context of recombinant protein expression, Dickson [42] has argued that the interpretation of these data sets can potentially assist in the identification or generation of the best producer cells, either via cell engineering and/or the optimization of media and feeds. Moreover, the raw materials can be controlled using metabolomics approaches and therefore, minimize batch-to-batch variations, as part of bioprocess development. This methodology is indeed

central for understanding CHO cell metabolism and, when employed in combination with other 'omics data, gives a snapshot of the cell's metabolic state.

4. Online Resources for Metabolism

In order to continually follow the updates in CHO metabolism and identify new growth-inhibiting and toxic metabolites, a number of online resources are helpful. www.CHOgenome.org is the access point for all publicly available genome-wide data of Chinese hamster and CHO cell lines [74]. Similarly, the CHOmine (https://chomine.boku.ac.at) is a data warehouse for CHO data with analysis tools. Additionally, CHOmine provides links to external websites and integrates recently published genome scale models (GEMs) [75]. Such models, developed by a consortium of researchers, allow for the integration 'omics data – genomics, transcriptomics, proteomics and metabolomics – for guiding hypothesis-driven discovery and metabolic engineering [76]. The GEMs are also excellent sources for an overview of CHO metabolites as models specifically developed for CHO cells exist and can support cell line engineering approaches and CHO cells' bioprocesses [77]. An earlier model [59] allowed for the identification of growth limiting factors and is available at http://CHO.sf.net (v1.1). More recently, the constraint-based models of Chinese hamster and CHO cell lines (CHO-S, CHO-K1 and CHO-DG44) were made available to researchers in the CHO field and can be downloaded from http://bigg.ucsd.edu/models/iCHOv1 and www.CHOgenome.org [77].

Additional useful databases are the metabolic database Kyoto Encyclopedia of Genes and Genome (KEGG) (http://www.kegg.jp), Reactome (http://www.reactome.org) and the Human Metabolome Database (HMDB) (http://www.hmdb.ca). KEGG provides information about metabolites and genes coding for enzymes which catalyze reactions participating in biochemical pathways [78–80]. Reactome is a tool for the visualization of the reactions, networks in the context of cellular compartments where anabolic and catabolic pathways occur [81]. Detailed information about small molecule metabolites that

are present in the human body [82–84] can be found in HMDB. This database can hint to which metabolites might affect CHO cells in culture, based on the toxic effects of the molecules to human cells, tissues, or organs.

In conclusion, the resources presented in Table 2 can aid and provide clues for medium development and for finding targets for engineering cells with improved phenotypes, based on the avoidance of unwanted metabolites.

5. Cell Line Engineering for Improved Nutrient Metabolism

Many strategies for cell line engineering have been employed in attempts to tackle the problematic of metabolic waste products which arise during cell culture, most extensively towards lactate production. The reviews by Ficher and colleagues [85] and Kim et al. [2] gather a number of cell line engineering approaches that were carried out in CHO cells. We briefly discuss cell line engineering approaches carried out in CHO cells which resulted in reduced lactate production. Reports have demonstrated the potential of engineering CHO cells, as seen in the case of the LdhA gene that was downregulated using RNAi technology, yielding reduced lactate production rates without impacting cell growth nor productivity of human thrombopoietin [86]. Similar results were observed with the downregulation of LdhA and pyruvate dehydrogenase kinase (Pdhk) isoenzymes 1, 2, and 3 in antibody producing-CHO cells [87]. However, the knockout of LdhA using zinc finger nucleases (ZFNs) in cells where Pdhk 1, 2, and 3 was down regulated was revealed to be lethal [88]. The overexpression of Aralar1, part of malate–aspartate shuttle (MAS), in a lactate-producing cell line led to a metabolic shift from lactate production to consumption [89]. This way, the authors found a link between MAS and this metabolic shift. Other investigational work involved the stable expression of fructose transporter (GLUT5) [90]. When cells used this sugar as a carbon source, the uptake rate of fructose was such (low) that the overflow of excess carbon to lactate was avoided. A number of research articles describe the effects of overexpressing the enzyme pyruvate carboxylase. The

overexpression of yeast pyruvate carboxylase (PYC2) resulted in a significant decrease in lactate production and increase in productivity [91]. An identical outcome was observed when human pyruvate carboxylase was engineered using a similar approach [92]. In a more recent study, the overexpression of codon optimized PYC2, reduced lactate production, and improved mAb production and glycosylation [93]. Additionally, improved cell metabolism was observed with the overexpression of malate dehydrogenase II (MDHII), which lead to an increase in intracellular ATP and NADH, and integral viable cell number [94]. The LC-MS analysis of the extracellular metabolites revealed the accumulation of malate, which was a result of an excess supply of aspartate in the medium and the presence of a bottleneck in MDHII in the TCA cycle.

More recently, the glutathione biosynthesis pathway was engineered through the stable overexpression of Gclc, which yielded increased GSH concentrations but did not improve productivity [95]. However, when the modifier subunit of Gcl was stably overexpressed in CHO host cells, an increase in specific productivity was observed once a mAb was transiently expressed by these cells. Surprisingly, the findings of this work allowed the conclusion that the GSH content does not contribute to the improvement of productivity of mAb in CHO cells, contrary to what was previously stated [68, 69].

Furthermore, the development of glutamine synthetase (GS) selection system exemplifies an important advance in recombinant protein expression using CHO cells, representing an alternative to dihydrofolate reductase (DHFR) expression system. The GS system is based on the knockout of the gene encoding for GS, which is reintroduced into the cell along with the vector encoding for recombinant protein [96]. The cells grow in glutamine free-medium under the selection pressure of methionine sulfoximine (MSX). An additional advantage of the GS system is that it allows for the reduction of by-product formation, as once the GS gene is reintroduced, ammonia along with glutamate is utilized to form of

glutamine. Glutamine becomes available for the formation of TCA cycle intermediates such as α-ketoglutarate.

6. Applications and future perspectives

In upstream process development for the production of recombinant therapeutic protein, both media and feed design, and cell line engineering can be employed. Cell line- or clone-specific media optimization may be required for each shortlisted candidate that is generated in one cell line development experiment, as these may display different growth phenotypes and by-product levels, as well as to account for the effects of clone-medium interactions [97].

The quantification of toxic metabolites in cell culture can aid the media development efforts, by indicating which precursor media and feed components are required to be supplied in controlled amounts. However, while media and feed optimization has enabled achievement of higher cell densities and increased productivity, it is still far from challenging the cell's maximized growth and production capacity that is predicted by metabolic network models. Metabolomics, along with other 'omics, provides an extra layer of knowledge on the cell metabolism and can lead to breakthroughs that improve these parameters. For instance, after the identification of toxic metabolic intermediates, such as the ones presented in Table 1, one can employ cell engineering tools to limit the formation of these inhibitory molecules. The metabolic pathways where these compounds are involved should be analyzed for identification of the target genes. Thereafter, it is essential to select the most suitable engineering strategy to perform specific genomic changes (e.g. downregulation or deletions of genes, or the overexpression of heterologous pathways that convert the toxic intermediates into "safer" molecules) for targeting genes encoding for enzymes forming such molecules. GEMs can be used to predict the effect of the transformation. The resulting phenotypic changes of the cell may indicate better nutrient usage and reduced the formation of toxic and inhibiting metabolites. Effective tools for

genome engineering, such as ZFN, and transcription activator-like effector nucleases (TALENs) employed in the past revealed themselves to be rather costly. The less costly and still efficient tool clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 system (CRISPR/Cas9) system (reviewed by Lee et al [98]) permits faster but yet specific gene targeting in mammalian cells. This genome editing tool offers new capabilities for streamlining CHO cell line development processes to obtain improved cell factories. A multiplexing cell engineering approach successfully reduced apoptosis and yielded non-fucosylated secreted proteins, through the simultaneous triple knockout of apoptotic Bcl-2 antagonist killer protein (BAK), Bcl-2-associated X protein (BAX), and fucosyltransferase 8 (Fut8) using CRISPR/Cas9 in CHO-S cells [99]. A similar approach can be employed to target metabolic genes.

Taken together – the metabolic models generated based on the integration of 'omics data, the employment of metabolomics for obtaining a detailed view of all active metabolic reactions in the cell and the recent genome editing tools which offer new capabilities for engineering and generating cells with ideal physiologic traits – form a well-connected trio which can enhance CHO cells factories as platforms for expression of therapeutic recombinant proteins.

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Conflict of interest

The authors declare no conflict of interests.

7. References

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Table 1. Metabolites with growth inhibitory effects, apoptosis inducers and waste products in CHO cell cultivations. Abbreviations: CDK, cyclin-dependent kinase; GS, glutamine synthetase; G3PC, Glycero-3-phosphocholine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamines; SEAP, Secreted embryonic alkaline phosphatase.

Metabolite	KEGG compound no.	Comment/effect	Pathways	Reference
Central/Energy metabolism				
Acetate	C00033	Acetate formed from acetyl-CoA starts to build up after the onset of the stationary phase.	Pyruvate metabolism	[43]
Citrate	C00158	Accumulation indicates TCA cycle truncation; increased along with alanine, upon supplementation of the medium with growth limiting nutrients (aspartate, asparagine, glutamate and pyruvate) in glutamine synthetase (GS) expression system. TCA cycle and fatty acid/lipid biosynthesis intermediates; related to mitchondria/cell redox status. In fed-batch, appeared in medium during culture in response to feed addition, representing changes in the mitochondria and changes in C-fluxes to alternative fates; Secreted during exponential phase	TCA cycle; Alanine, aspartate and glutamate metabolism	[26] [39] [43]
Fructose	C00085	Increased intracellularly after addition of feed containing glucose; build up may occur in connection to sorbitol.	Sorbitol pathway	[33]
Fumarate	C00122	Secreted during exponential phase	TCA cycle; Alanine, aspartate and glutamate metabolism	[43]
Lactate	C00186	Inhibits cell growth; Accumulation results in lowered pH and changes in osmolarity due to the presence of base, added to counter the effects of decreased pH from lactate formation. Reduces cell growth due to acidification; reported to inhibit cell growth in cultures that do not employ pH control in murine hybridoma cell line. When present in the cell culture medium, reduces growth and induces cell death in baby hamster kidney cells. Accumulation in medium is linked to growth phase of culture.	Pyruvate metabolism	[17] [27] [18] [25] [26]
Malate	C00149	Accumulated extracellularly; linked to aspartate supplied in the medium and to enzymatic bottleneck at malate dehydrogenase II in TCA cycle. Accumulated in medium during a fed-batch culture in response to feed addition; represents changes in the mitochondria and changes in C-fluxes to alternative fates;	TCA cycle; Pyruvate metabolism	[94] [39]

		Secreted during exponential phase		[43]
Sorbitol	C00794	Released into the medium and represents carbon losses to the cell; alternative redox sink for the cell; linked to the cellular redox state (NADPH/NADP+) and inform of cell well-being during culture. Linked to the cellular redox state (NADPH/NADP+). Builds up intracellularly in cells growing media containing high and low copper, related to metabolic shift from lactate production to lactate consumption phenotype	Fructose and mannose metabolism; Galactose metabolism	[26] [39] [33]
Succinate	C00042	Secreted during exponential phase	TCA cycle, Oxidative phosphorylation, Alanine, aspartate and glutamate metabolism	[43]
Threitol	C16884	Linked to the cellular redox state (NADPH/NADP+) and is an alternative redox sink for the cell.		[39]
Amino acid metabolism				
Alanine	C00041	Accumulated in the medium, negative effect in cell growth; Inhibits pyruvate kinase and TCA pathway; potential source of ammonia Produced during culture. Formed by transamination from pyruvate; Accumulated in the medium along with glycine and citrate in the transition of exponential phase to stationary Produced from pyruvate at late stages of culture. Accumulated in the medium	Alanine, Aspartate and Glutamate metabolism	[53] [39] [26] [42] [43]
Ammonia	C00014	Decreases specific cell growth rate, increases consumption rates of glucose and glutamine and decrease antibody product titer in hybridoma cells. Affects intracellular pH, cell growth and recombinant protein productivity, and product glycosylation. Reduces of growth rates and maximal densities, changes metabolic rates, affects protein processing in mammalian cells. Production of ammonia and alanine is linked to the consumption of asparagine and glutamine in a GS-CHO cell line.	Amino acid metabolism	[18] [100] [20] [53]
Asparagine	C00152	Asparagine consumption has been correlated with accumulation of ammonia and alanine. Highest consumed amino acid in GS-CHO cells treated with butyrate	Alanine, Aspartate and Glutamate metabolism	[59] [43]
Glutamine	C00064	Degradation of glutamine generates ammonium and glutamate. Extracellular supply of glutamine and pyruvate are sources of lactate formation.	Glutamate metabolism	[48] [100] [101]

Glycine	C00037	Product of serine catabolism Accumulation in the medium indicates a positive effect. Accumulated in the medium along with alanine, in the transition of exponential phase to stationary Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition. Accumulation of glycine is beneficial for the cells due to its role in GSH biosynthesis.	Glycine, Serine and Threonine metabolism	[43] [53] [26] [57] [58] [59]
Leucine	C00123	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Valine, Leucine and Isoleucine metabolism	[57] [58]
Lysine	C00047	Oversupplied nutrient; accumulates in the medium during death phase.	Lysine metabolism	[56]
Methionine	C00073	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Cysteine and methionine metabolism	[57] [58]
Phenylalanine	C00079	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Phenylalanine, Tyrosine and Tryptophan metabolism	[57] [58]
Serine	C00065	Highly consumed amino acid in GS-CHO cells treated with butyrate Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Glycine, Serine and Threonine metabolism	[43] [57] [58]
Threonine	C00188	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Glycine, Serine and Threonine metabolism	[57] [58]
Tryptophan	C00078	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Phenylalanine, Tyrosine and Tryptophan metabolism	[57] [58] [57]
Tyrosine	C00082	Recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Phenylalanine, Tyrosine and Tryptophan metabolism	[57] [58] [57]
Amino acid derivatives				
Dimethylarginine (DARG)	C03626	Accumulates in the media over culture time; linked to excessive supply of Arginine. Induces apoptosis in endothelial cells due to intracellular oxidant production and related to p38 mitogen-activated protein kinase (MAPK)/caspase-3-dependent signaling pathway. Known to induce apoptosis in human endothelial cells, by increasing intracellular reactive oxygen species production	Arginine metabolism	[59] [102] [103]
Formate	C00058	Product of serine catabolism Product of serine catabolism, growth inhibitory at concentrations between 4-10 mM. Metabolic by-product; recommended to keep concentration bellow 2 mM in fed-	Glycine, serine and threonine metabolism	[43] [104] [57] [58]

		batch process due to growth inhibition.		
Homocysteine	C00155	Metabolic by-product; recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Cysteine and Methionine metabolism	[57] [58]
Indole-3-carboxylate	C19837	Metabolic by-product; recommended to keep concentration bellow 1 mM in fed-batch process due to growth inhibition.	Tryptophan metabolism	[57] [58]
Indolelactate	C02043	Metabolic by-product; recommended to keep concentration bellow 3 mM in fed-batch process due to growth inhibition.	Tryptophan metabolism	[57] [58]
Isobutyrate	C02632	Accumulated in culture as result of breakdown of the branched-chain amino acids.	Valine metabolism	[43]
Isovalerate	C08262	Accumulated in culture as result of breakdown of the branched-chain amino acids. Metabolic by-product; recommended to keep concentration bellow 1 mM in fed-batch process due to growth inhibition.	Leucine metabolism	[43] [58] [57]
Methylglyoxal	C00546	Detrimental to cultured cells; D-lactic acid is the end product of methylglyoxal metabolism in mammalian cells; Inhibits cell growth and induces apoptosis when added to the medium in hybridoma cell cultures; By-product formed through non-enzymatic decomposition of dihydroxyacetone phosphate and glyceraldehyde-3-phophate;	Glycine, serine and threonine metabolism; Pyruvate metabolism Glycolysis	[105] [65] [106]
Ornithine	C00077	Present in death phase of culture and associated with apoptosis.	Arginine and proline metabolism	[56] [107]
Phenyllactate	C05607	Metabolic by-product; recommended to keep concentration bellow 1 mM in fed-batch process due to growth inhibition.	Phenylalanine metabolism	[57] [58]
2-hydroxybutyric acid	C05984	Metabolic by-product; recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Cysteine and methionine metabolism	[57] [58]
3-(4-hydroxyphenyl)lactate	C03672	Metabolic by-product; recommended to keep concentration bellow 0,5-1 mM in fed-batch process due to growth inhibition.	Phenylalanine, Tyrosine and Tryptophan metabolism	[57] [58]
4-hydroxyphenylpyruvate	C01179	Metabolic by-product; recommended to keep concentration bellow 1 mM in fed-batch process due to growth inhibition.	Phenylalanine, Tyrosine and Tryptophan metabolism	[57] [58]
Nucleotide metabolism				
Adenosine	C00212	Cytotoxic: induces apoptosis in cells of the immune system, nervous system and endothelium. Increased metabolic capacity of the cell.	Purine metabolism; Signaling pathways	[40]
ADP	C00008	Arrests cell cycle in G1 in CHO cells overexpressing p27, a cyclin-dependent kinase (CDK) inhibitor, and increased Secreted embryonic alkaline phosphatase (SEAP) specific productivity. Increased metabolic capacity of the cell.	Purine metabolism; Oxidative phosphorylation	[108] [40]
Adenosine monophosphate (AMP)	C00020	Cytotoxic: induces apoptosis when added to cell culture medium at 2mM, in at lower concentrations (1mM) arrests cell growth and increases productivity. Increased metabolic capacity of the cell.	Purine metabolism; Signaling pathways	[40]

		Arrests cell cycle in G1 in CHO cells overexpressing p27, a CDK inhibitor, and increased SEAP specific productivity. Addition to fresh CHO mAb cultures lead to apoptosis.		[108] [41]
Adenosine triphosphate (ATP)	C00002	Cytotoxic: induces apoptosis in cells of the immune system, nervous system and endothelium. Increased metabolic capacity of the cell. Arrests cell cycle in G1 in CHO cells overexpressing p27, a CDK inhibitor, and increased SEAP specific productivity.	Purine metabolism; Oxidative phosphorylation	[40] [108]
guanosine diphosphate (GDP)	C00035	Leads to cell growth arrest.	Purine metabolism, Signaling pathways	[40]
Guanosine monophosphate (GMP)	C00144	Added to culture medium and decreased cell growth. This effect was shown not to be cell line dependent. May improve protein production after arresting cell growth. Addition to fresh CHO mAb cultures lead to apoptosis.	Purine metabolism, Signaling pathways	[40] [41]
Lipid metabolism				
Choline phosphate (PCHO)	C00588	Depleting over time (144h) in fed-batch cultivation; linked to the build-up of extracellular G3PC and to cell growth limitation. By-product of choline, builds up after 72 h of cultivation.	Glycerophospholipid metabolism	[59] [43]
Glycerol	C00116	Possibly formed from glycerol-3 phosphate; Released into the medium and represent carbon losses to the cell; Linked to the cellular redox state (NADH/NAD+) and inform of cell well-being during culture. Glycerol accumulated over culture time, as a result of branching from glycolysis at dihydroxyacetone-phosphate (DHAP) with NADH oxidation. Alternative redox sink and related to mitochondria/cell redox status	Glycerolipid metabolism	[26] [42] [43] [39] [42]
Glycerol-3-phosphate	C00093	Builds up intracellular concentration and these changes are related to phospholipid synthesis and cell growth.	Glycerolipid metabolism; Glycerophospholipid metabolism	[26] [42]
Glycero-3-phosphocholine (G3PC)	C00670	Builds-up over time as intracellular precursors of PE and PC deplete; linked to cell growth limitation. By-product of choline, builds up after 72 h of cultivation.	Glycerophospholipid metabolism	[59] [43]
Redox metabolites				
GSSG	C00127	Addition to fresh CHO mAb cultures lead to apoptosis. Linked to oxidative stress; potential growth-limiting factor. Accumulates extracellularly towards the end of the culture.	Glutathione metabolism	[41] [59]

Table 2. Summary of publicly available resources relevant for CHO research.

Name	Description/Comment	URL	Reference
CHOgenome	Host of all published CHO-related data. Compiles genome-scale information of Chinese hamster and CHO-K1.	http://www.chogenome.org	[74]
CHOmine	Data warehouse for CHO data and provides links to outside websites containing information on gene and protein. It integrates the recently published genome scale model for *C. griseus.* and CHO cell lines.	https://chomine.boku.ac.at	[75]
Genome scale model for *C. griseus.* and CHO cell lines	Genome scale model of global model of Chinese hamster (*C. griseus)* metabolism and cell line-specific models of CHO-S, K1, and DG44.	http://bigg.ucsd.edu/models/iCHOv1	[77]
Standardized network reconstruction of CHO cell metabolism	Genome-scale network reconstruction of CHO cell metabolic network, as based on genome sequence and literature.	CHO.sf.net (v1.1)	[59]
Kyoto Encyclopedia of Genes and Genome (KEGG)	Metabolic database that provides information about metabolites and genes coding for enzymes catalyzing reactions part of metabolic pathways. Data accessible for several organisms including *C. griseus*.	http://www.kegg.jp	[80] [79] [78]
Reactome Knowledgebase	Archive of biological processes and a tool for discovering functional relationships in data; Provides visualization of reaction networks and details of single reactions; some *N*-glycosylation pathways for *C. griseus* are available and other metabolic maps are not fully accessible. Therefore, we recommend using this tool based on well annotated and closely related organisms, such as *H. sapiens* or *M. musculus*, as a guide.	http://www.reactome.org	[81]
Human metabolome database	The Human Metabolome Database (HMDB) contains detailed information about small molecule metabolites found in the human body. It is intended to be used for applications in metabolomics among others. Additionally, it provides chemical data, clinical data, and molecular biology/biochemistry data.	http://www.hmdb.ca	[84] [83] [82]

Figure legends

Figure 1. Schematic of main biosynthetic and catabolic pathways of CHO cells linked to production of toxic or inhibiting compounds. See Table 1 for details on individual metabolites.

Figure 2. Glutathione biosynthesis and cycling based on the genes present in Chinese hamster (*C. griseus*) genome, derived from KEGG pathways (http://www.kegg.jp). Glutathione (GSH) biosynthesis occurs in the cytosol using glutamate, cysteine and glycine as precursors. The first reaction is catalyzed by the enzyme Gcl, which is composed by two subunits: Gcl catalytic subunit (Gclc) and Gcl regulatory subunit (Gclm), followed by a reaction catalyzed by Gss. Reactions of GSH with ROS are mediated by enzymes from the Gst family. Abbreviations: ATP – adenosine triphosphate, Gcl – glutamamte cysteine ligase, Gss – glutathione synthetase. Ggct – gamma-glutamylcyclotransferase, Gpx* – glutathione peroxidase family, Txndc12 – thioredoxin domain containing 12, Gsr – glutathione-disulfide reductase, G6dp – glucose-6-phosphate dehydrogenase, Gstp* – glutathione S-transferase family, Anpep – alanyl aminopeptidase membrane, Lap3 – leucine aminopeptidase 3, Cth – cystathionine gamma-lyase, Ggt* – gamma-glutamyltransferase type enzymes. "?" – reaction (represented with dashed line arrow) required for the endogenous formation of cystine, not present in *C. griseus* genome according to the consulted database.

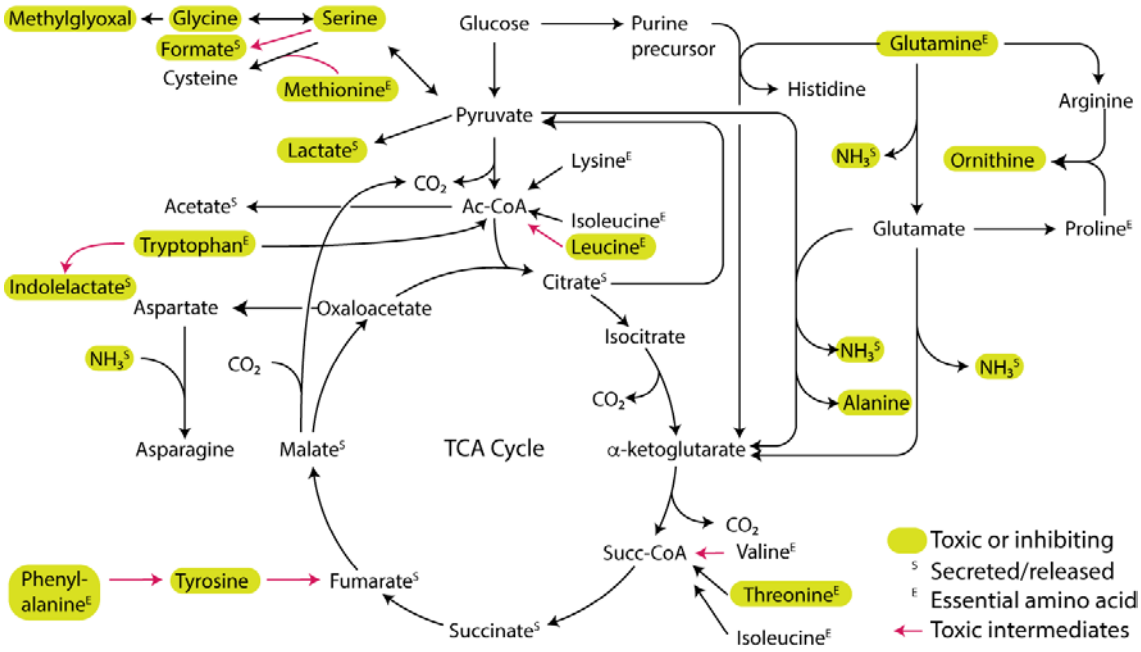

Figure 1

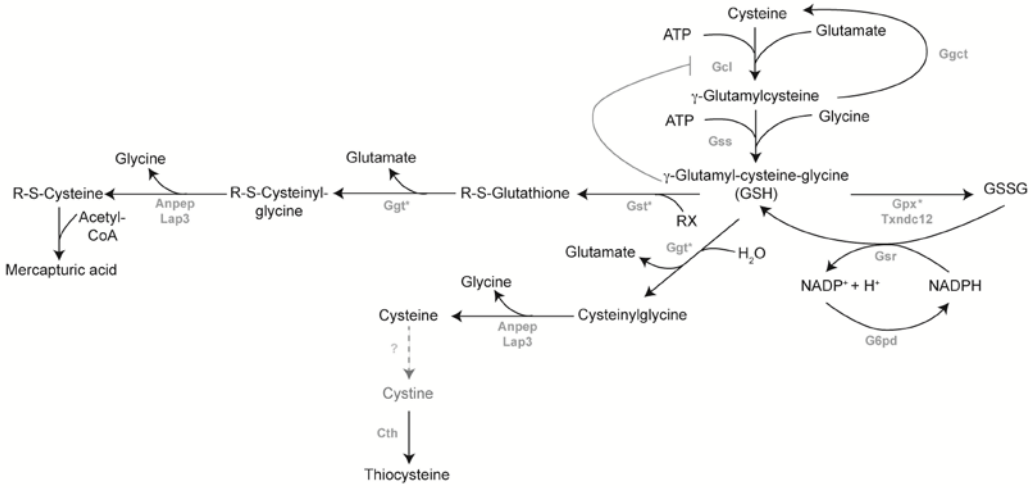

Figure 2