



UNIVERSIDAD NACIONAL DE COLOMBIA

1       Identifying proteins and metabolic  
2       pathways associated with the  
3       neuroprotective response mediated by  
4       tibolone in astrocytes under an  
5       induced inflammatory model.

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## Resumen

En este trabajo, se identificaron las proteínas y rutas metabólicas asociadas a la respuesta neuroprotectora mediada por el neuroesteroide sintético tibolona bajo un modelo inflamatorio inducido por palmitato usando análisis de balance de flujo (FBA). Para tal fin, se modelaron tres diferentes escenarios metabólicos ('saludable', 'inflamado' y 'medicado') bajo una reconstrucción metabólica tejido-específica de astrocitos maduros construida a partir de datos de expresión génica. La reconstrucción metabólica se construyó, validó y limitó usando tres paquetes de software ('minval', 'g2f' y 'exp2flux') liberados a través de los repositorios R CRAN durante el desarrollo de este trabajo. A partir de nuestros análisis, predecimos que la tibolona ejecuta sus acciones neuroprotectoras a través de la reducción de la neurotoxicidad mediada por el L-glutamato en astrocitos, induciendo la activación de varias rutas metabólicas con funciones neuroprotectoras asociadas como: Metabolismo de taurina, gluconeogénesis y rutas de señalización PPAR y mediadas por calcio. Adicional a esto, encontramos un aumento en la tasa de crecimiento asociado a la tibolona que podría estar relacionado con efectos secundarios reportados para los compuestos esteroideos en otros tipos celulares humanos.

**Palabras clave:** Astrocitos, Tibolona, Neuroprotección, Inflamación, Análisis de Balance de Flujo.

## Abstract

In this work, proteins and metabolic pathways associated with the neuroprotective response mediated by the synthetic neurosteroid tibolone under a palmitate-induced inflammatory model were identified by flux balance analysis (FBA). Three different metabolic scenarios ('healthy', 'inflamed' and 'medicated') were modeled over a gene expression data-driven constructed tissue-specific metabolic reconstruction of mature astrocytes. Astrocyte reconstruction was built, validated and constrained using three open source software packages ('minval', 'g2f' and 'exp2flux') released through CRAN R repositories during the development of this work. From our analysis, we predict that tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes, inducing the activation several metabolic pathways with neuroprotective actions associated such as taurine metabolism, gluconeogenesis, calcium and PPAR signaling pathways. Also, we found a tibolone associated increase in growth rate probably in concordance to previously reported side effects of steroid compounds in other human cell types.

**Keywords:** Astrocytes, Tibolone, Neuroprotection, Inflammation, Flux Balance Analysis.



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129

# Objectives

130

## Main:

131

Identify proteins and metabolic pathways involved in the neuroprotective effects of tibolone in human astrocytes based on metabolic scenarios comparison.

132

133

## Specific

134

- Build a tissue-specific computational model of astrocytes metabolism using gene expression data integration.

135

136

- Evaluate the effects caused by the increase of free fatty acids and tibolone presence in astrocytes metabolism.

137

138

- Determine metabolic pathways and relevant functional products in response to steroid tibolone through systems biology approximations.

139

140

- Evaluate the importance of proteins and metabolic pathways previously identified on the dynamics of the metabolic model.

141

# 1. Building a metabolic reconstruction: Doing a MINimal VALidation of stoichiometric reactions through 'minval' R package.

**Original title:** minval: An R package for MINimal VALidation of stoichiometric reactions.

**Written by:** *Daniel Osorio, Janneth Gonzalez and Andrés Pinzón-Velasco*

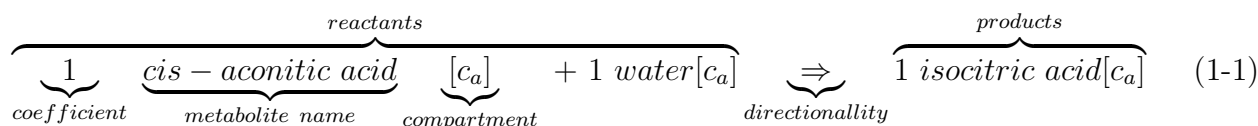
## Abstract

The genome-scale metabolic reconstructions, a compilation of all stoichiometric reactions that can describe the entire cellular metabolism of an organism, have become an indispensable tool for our understanding of biological phenomena, covering fields that range from systems biology to bioengineering. Evaluation of metabolic reconstructions are generally carried through Flux Balance Analysis, an optimization method where the biological sense of optimal solution is sensitive to thermodynamic unbalance, caused by the presence of stoichiometric reactions whose compounds are not produced or consumed in any other reaction (orphan metabolites) and by mass unbalanced stoichiometric reactions. The **minval** package was designed as a tool to identify orphan metabolites and the mass unbalanced reactions in a set of stoichiometry reactions, it also permits to extract all reactants, products, metabolite names and compartments from a metabolic reconstruction, moreover specific functions to map compound names associated to the Chemical Entities of Biological Interest (ChEBI) database are also included.

## 1.1. Introduction

A chemical reaction is a process where a set of chemical compounds called *reactants* are transformed into another compounds called *products* [1]. The accepted way to represent a chemical reaction is called a *stoichiometric reaction*, where reactants are placed on the left and the products on the right separated by an arrow which indicates the direction of the

166 reaction as is showed in the equation 1-1 [2]. In biochemistry a set of chemical reactions  
167 that transform a substrate into a product, after several chemical transformations is called a  
168 metabolic pathway [3]. The compilation of all stoichiometric reactions included in all meta-  
169 bolic pathways that can describe the entire cellular metabolism encoded in the genome of a  
170 particular organism is known as a *genome-scale metabolic reconstruction* [4] and has become  
171 an indispensable tool for studying metabolism of biological entities at the systems level [5].  
172



173  
174  
175 Reconstruction of genome-scale metabolic models starts with a compilation of all known  
176 stoichiometric reactions for a given organism, as evidenced by the presence of enzyme coding  
177 genes in its genome. Thus the reactions in which these enzymes are known to participate  
178 in, are usually downloaded from specialized databases as KEGG [6], BioCyc [7], Reacto-  
179 me [8], BRENDA [9] and SMPDB [10], however the downloaded stoichiometric reactions  
180 are not always mass-charge balanced and don’t represent complete pathways to construct a  
181 high-quality metabolic reconstruction [5, 11]. The identification and curation of these type  
182 of reactions is a time consuming process which the researcher have to complete manually  
183 using available literature or experimental data [12].  
184

185 Genome-scale metabolic reconstructions are usually interrogated through *Flux Balance Analy-*  
186 *sis* (FBA), an optimization method that allows us to understand the metabolic status of the  
187 cell, improve the production capability of a desired product or make a rapid evaluation of  
188 cellular physiology at genome-scale [4, 13]. Nevertheless FBA is sensitive to thermodynamic  
189 unbalance, so in order to asses the validity of a biological extrapolation (i.e. an optimal  
190 solution) from a FBA analysis it is mandatory to avoid this type of unbalancing in mass  
191 conservation through all model reactions [14]. Another drawback when determining the vali-  
192 dity of a metabolic reconstruction is the presence of reactions with compounds that are not  
193 produced or consumed in any other reaction (dead ends), generally known as orphan meta-  
194 bolites [4, 5]. The presence of this type of metabolites can be problematic, since they lead to  
195 an artificial cellular accumulation of metabolism products which therefore bias our biological  
196 conclusions. Tracking these metabolites is also a time consuming process, which most of the  
197 time has to be performed manually or partially automatized by in-house scripting. Given  
198 that typical genome-scale metabolic reconstructions account for hundreds or thousands of  
199 biochemical reactions, the manually curation of these models is a task that can lead to both,  
200 the introduction of new errors and to overlook some others.

201  
202 The most popular FBA implementations as COBRA and RAVEN includes similar functions

(`checkMassChargeBalance` and `getElementalBalance` respectively) implemented under the commercial MATLAB<sup>®</sup> environment. These functions identify orphan metabolites and mass unbalanced reaction based in the chemical formula or the IUPAC International Chemical Identifier (InChI) supplied manually by the user for each metabolite included in the genome-scale metabolic reconstruction. With the aim to automatize the identification of orphan metabolites as well as the unbalanced stoichiometric reactions in a genome-scale metabolic reconstruction, we have developed the **minval** package. It includes thirteen functions to evaluate mass balance and extract all reactants, products, orphan metabolites, metabolite names and compartments for a set of stoichiometric reactions, moreover specific functions to map compound names associated to the Chemical Entities of Biological Interest (ChEBI) database are also included.

For this version we use the included “glugln” dataset [15], 128 non-exchange/sink stoichiometric reactions from the reconstruction of the glutamate/glutamine cycle constructed in-house using the KEGG database, as an example for each function included in the **minval** package with the aim to show their potential use.

## 1.2. Installation and functions

**minval** includes 15 functions and is available for download and installation from CRAN, the Comprehensive R Archive Network. To install and load it, just type:

```
> install.packages("minval")  
> library(minval)
```

The **minval** package requires R version 2.10 or higher. Development releases of the package are available on the GitHub repository <http://github.com/gibbslab/minval>.

### Inputs and syntaxis

The functions included in **minval** package take as input a string list with stoichiometric reactions. The data loading from traditional human-readable spreadsheets can be carried through other CRAN-available packages as **gdata**, **readxl** or **xlsx**. Each reaction string must contain metabolites, with an optional compartment label between square brackets. The metabolites should be separated by a plus symbol (+) between two blank spaces and may have just one stoichiometric number before the name. The reactants should be separated of products by an arrow using the following symbol  $\Rightarrow$  for irreversible reactions and  $\Leftrightarrow$  for reversible reactions.

## 233 Syntax Validation

234 Flux Balance Analysis method is implemented in a variety of software under different pro-  
235 gramming languages. Some of the most popular implementations are COBRA [16] and RA-  
236 VEN [17] under matlab language as well as sybil and abcdeFBA under R language. The  
237 isValidSyntax function validate the well accepted compartmentalized stoichiometric syn-  
238 tax (Equation 1-1) for several FBA implementations and returns a boolean value TRUE if  
239 syntax is correct. In this example we show the stoichiometric syntax for the inter-conversion  
240 of malate to fumaric acid and water in astrocytes cytoplasm.

```
> isValidSyntax("(S)-malate(2-)[c_a] <=> fumaric acid[c_a] + water[c_a]")
[1] TRUE
```

## 241 Reactants and Products

242 As defined in introduction, stoichiometric reactions represent the transformation of reactants  
243 into products in a chemical reaction. The reactants and products functions extract and  
244 return all reactants and products present in a stoichiometric reaction as a vector. In this  
245 example we show the extraction of the reactants (quinone and succinic acid) and products  
246 (hydroquinone and fumaric acid) in a reaction that occurs in astrocytes mitochondrias.

```
> reactants("Quinone[m_a] + succinic acid[m_a] => Hydroquinone[m_a] + fumaric acid[m_a]")
[1] "Quinone[m_a]"      "succinic acid[m_a]"
> products("Quinone[m_a] + succinic acid[m_a] => Hydroquinone[m_a] + fumaric acid[m_a]")
[1] "Hydroquinone[m_a]" "fumaric acid[m_a]"
```

## 247 Metabolites

248 Two of the more popular packages that implement FBA analysis such as COBRA [16] and RAVEN  
249 [17] require the complete list of metabolites included in the metabolic reconstruction, in a  
250 particular section of the human-readable input file. The metabolites function automatically  
251 identifies and lists all metabolites (with and without compartments) for a specific or a set of  
252 stoichiometric reactions. In this example we show how to extract all metabolites (reactants  
253 and products) with and without compartment for the Ubiquinol and FAD production reaction  
254 in astrocytes mitochondrias.

```
> metabolites("FADH2[m_a] + ubiquinone-0[m_a] => FAD[m_a] + Ubiquinol[m_a]")
[1] "FADH2[m_a]"      "ubiquinone-0[m_a]" "FAD[m_a]"
[4] "Ubiquinol[m_a]"
```

255 As was mentioned before, the report option without compartment was added:

```
> metabolites("FADH2[m_a] + ubiquinone-0[m_a] => FAD[m_a] + Ubiquinol[m_a]",
+             woCompartment = TRUE)
[1] "FADH2"           "ubiquinone-0" "FAD"           "Ubiquinol"
```

## 256 Orphan Metabolites

257 Orphan metabolites, compounds that are not produced or consumed in any other reaction are  
 258 one of the main causes of mass unbalance in metabolic reconstructions. The **orphanReactants**  
 259 function, identifies compounds that are not produced internally by any other reaction and  
 260 should be added to the reconstruction as an exchange reaction following the protocol propo-  
 261 sed by [5]. In this examples we show how to extract all orphan compounds for all reactions  
 262 included in the glutamate/glutamine cycle.

```
> data("glugln")
> orphanReactants(glugln)

[1] "alpha-D-Glucose 6-phosphate[r_n]" "water[r_n]"
[3] "2,3-bisphospho-D-glyceric acid[r_n]" "GTP[c_n]"
[5] "oxaloacetic acid[m_n]" "citric acid[c_n]"
[7] "coenzyme A[c_n]" "Quinone[m_n]"
[9] "D-Glutamine[m_n]" "L-Glutamine[m_n]"
[11] "FADH2[m_n]" "oxygen atom[m_n]"
[13] "Ferrocycytochrome c2[m_n]" "diphosphate(4-)[m_n]"
[15] "alpha-D-Glucose 6-phosphate[r_a]" "water[r_a]"
[17] "2,3-bisphospho-D-glyceric acid[r_a]" "GTP[c_a]"
[19] "hydrogencarbonate[m_a]" "citric acid[c_a]"
[21] "coenzyme A[c_a]" "Quinone[m_a]"
[23] "L-glutamic acid[c_a]" "Ammonia[c_a]"
[25] "FADH2[m_a]" "oxygen atom[m_a]"
[27] "Ferrocycytochrome c2[m_a]" "diphosphate(4-)[m_a]"
```

263 The **orphanProducts** function, identifies compounds that are not consumed internally by  
 264 any other reaction and should be added to the reconstruction as an sink reaction following  
 265 the protocol proposed by [5]. In this example we show the option added to **orphan\*** functions,  
 266 that permits to report the orphan metabolites as a list grouped by compartment:

```
> orphanProducts(glugln, byCompartment = TRUE)

$r_n
[1] "alpha-D-Glucose[r_n]" "phosphate(3-)[r_n]"
[3] "2-phospho-D-glyceric acid[r_n]"

$c_n
[1] "GDP[c_n]" "(S)-Lactate[c_n]" "acetyl-CoA[c_n]"

$m_n
[1] "Hydroquinone[m_n]" "D-glutamic acid[m_n]"
[3] "FAD[m_n]" "Ferricytochrome c2[m_n]"

$r_a
[1] "alpha-D-Glucose[r_a]" "phosphate(3-)[r_a]"
[3] "2-phospho-D-glyceric acid[r_a]"
```

```
$c_a
[1] "GDP[c_a]" "(S)-Lactate[c_a]" "acetyl-CoA[c_a]" "L-Glutamine[c_a]"

$m_a
[1] "Hydroquinone[m_a]" "FAD[m_a]"
[3] "Ferricytochrome c2[m_a]"
```

## 267 Compartments

268 As well as in cells, where not all reactions occur in all compartments, stoichiometric reactions  
269 in a metabolic reconstruction can be labeled to be restricted for a single compartment during  
270 FBA, by the assignment of a compartment label after the stoichiometric coefficient and  
271 name of each metabolite. Some FBA implementations require the report of all compartments  
272 included in the metabolic reconstruction as an independent part of the human-readable input  
273 file. In this example we show how to extract all compartments for all reactions included in  
274 the glutamate/glutamine cycle.

```
> compartments(glugln)

[1] "c_n" "r_n" "m_n" "c_a" "r_a" "m_a"
```

## 275 Association with ChEBI

276 The Chemical Entities of Biological Interest (ChEBI) database is a freely available dictionary  
277 of molecular entities focused on ‘small’ chemical compounds involved in biochemical reactions  
278 [18]. Amongst other characteristics, the release 136 of ChEBI database contains a set of  
279 standardized metabolite names, synonyms and molecular formula for at least 52521 chemical  
280 compounds. The use of standardized metabolite names facilitate the sharing process and  
281 inter-conversion to another metabolite names or identifiers [19, 20]. The minval package  
282 contains five functions to check and extract values from a local copy of the ChEBI database  
283 release 136. The `is.chebi` function takes a compound name as input, compares it against  
284 all the compounds names in ChEBI and returns a logical value TRUE if a match is found.  
285 In this next four examples we show the potential use of the functions using as input the  
286 acetyl-CoA compound.

```
> is.chebi("acetyl-CoA")

[1] TRUE
```

287 The `chebi.id` function takes a compound name as input, compares it against all the com-  
288 pounds names in ChEBI and returns the compound identifier if a match is found.

```
> chebi.id("acetyl-CoA")
```



```
[1] "15351"
```

289 The `chebi.formula` function takes a compound name as input, compares it against all the  
290 compounds names in ChEBI and returns the molecular formula if a match is found.

```
> chebi.formula("acetyl-CoA")
```

```
[1] "C23H38N7O17P3S"
```

291 The `chebi.candidates` function takes a compound name as input, compares it against all  
292 the compounds synonyms in ChEBI and returns possible compound names if a match is  
293 found.

```
> candidates<-chebi.candidates("acetyl-CoA")
> head(candidates)
```

```
[1] "acetoacetyl-CoA"          "acetyl-CoA"
[3] "(1-hydroxycyclohexyl)acetyl-CoA" "cinnamoyl-CoA"
[5] "2-methylacetoacetyl-CoA"    "phenylacetyl-CoA"
```

294 The `to.ChEBI` function translates the compounds names of a stoichiometric reaction into  
295 their corresponding identifier or molecular formula in the ChEBI database. In this example  
296 we show how to use the `to.ChEBI` function for the Ubiquinol and FAD production reaction  
297 in astrocytes mitochondrias.

```
> toChEBI("FADH2[m_a] + ubiquinone-0[m_a] => FAD[m_a] + Ubiquinol[m_a]")
```

```
[1] "1 17877 + 1 27906 => 1 16238 + 1 17976"
```

```
> toChEBI("FADH2[m_a] + ubiquinone-0[m_a] => FAD[m_a] + Ubiquinol[m_a]",formula = TRUE)
```

```
[1] "1 C27H35N9O15P2 + 1 C9H10O4 => 1 C27H33N9O15P2 + 1 C9H12O4(C5H8)n"
```

## 298 Mass Balance Validation

299 Thermodynamic unbalance of genome-scale metabolic reconstructions can also be promo-  
300 ted by stoichiometric mistakes. In a well balanced stoichiometric reaction according to the  
301 Lomonósov-Lavoisier law, the mass comprising the reactants should be the same mass present  
302 in the products. The `isBalanced` function converts the metabolites identifiers to molecular  
303 formulas, multiplies the atom numbers by their respective stoichiometric coefficient, and es-  
304 tablishes if the atomic composition of reactants and products are the same, it returns a logical  
305 value TRUE if mass is unbalanced. In this example we show the mass balance evaluation for  
306 the first twenty reactions of the glutamate/glutamine cycle.

```
> isBalanced(glugln[1:20])
```

```
[1] FALSE TRUE FALSE FALSE FALSE FALSE FALSE FALSE FALSE FALSE
[13] FALSE TRUE FALSE FALSE TRUE TRUE TRUE TRUE
```

307 The `isBalanced` function also include an option to show the molecular formula of mass  
308 unbalanced formulas through the option `show.formulas`.

```
> isBalanced(glugln[1:20], show.formulas = TRUE)

[,1]
[1,] "alpha-D-Glucose 6-phosphate[r_n] + water[r_n] => alpha-D-Glucose[r_n] + phos ..."
[2,] "beta-D-fructofuranose 1,6-bisphosphate[c_n] + water[c_n] => beta-D-fructofur ..."
[3,] "D-Glyceraldehyde 3-phosphate[c_n] + phosphate(3-)[c_n] + NAD(+)[c_n] <=> 3-p ..."
[4,] "ATP[c_n] + 3-phosphoglyceric acid[c_n] <=> ADP[c_n] + 3-phosphonato-D-glycer ..."
[5,] "3-phosphonato-D-glyceroyl phosphate(4-)[c_n] => 2,3-bisphospho-D-glyceric ac ..."
[6,] "2,3-bisphospho-D-glyceric acid[c_n] + water[c_n] => 3-phosphoglyceric acid[c ..."
[,2]
[1,] "1 C6H13O9P + 1 H2O => 1 C6H12O6 + 1 O4P"
[2,] "1 C6H14O12P2 + 1 H2O => 1 C6H13O9P + 1 O4P"
[3,] "1 C3H7O6P + 1 O4P + 1 C21H28N7O14P2 <=> 3 C3H4O10P2 + 1 C21H29N7O14P2 + 1 H"
[4,] "1 C10H16N5O13P3 + 3 C3H7O7P <=> 1 C10H15N5O10P2 + 3 C3H4O10P2"
[5,] "3 C3H4O10P2 => 2 C3H8O10P2"
[6,] "2 C3H8O10P2 + 1 H2O => 3 C3H7O7P + 1 O4P"
```

### 309 1.3. Summary

310 We introduced the `minval` package to evaluate mass balancing correctness of metabolic re-  
311 constructions and to extract all reactants, products, orphan metabolites, metabolite names  
312 and compartments for a set of stoichiometric reactions. We show step by step the minimal  
313 evaluation process of mass balance using the 128 non-exchange reactions included in the glu-  
314 tamate/glutamine cycle included in the “`glugln`” dataset. Also some examples of metabolites  
315 names - ChEBI database association was showed.

## 2. Building a tissue-specific metabolic reconstruction: Finding and filling gaps in metabolic networks through ‘g2f’ R package.

**Original title:** g2f: An R package for find and fill gaps in metabolic networks.  
**Written by:** Kelly Botero, Daniel Osorio, Janneth Gonzalez and Andrés Pinzón-Velasco

### Abstract

During the building of a genome-scale metabolic network reconstruction, several dead-end metabolites which cannot be imported/produced, or that are not used as substrates or released by not any of the reactions are incorporated into the network. The presence of dead-end metabolites can block out the net flux of the objective function when is evaluated through Flux Balance Analysis (FBA), and when it is not blocked, bias in the biological conclusions increase. The refinement to restore the connectivity of the network can be carried out manually or using computational algorithms. The **g2f** package was designed as a tool to find the dead-end metabolites, and fill it from the stoichiometric reactions of a reference, filtering candidate reactions using a weighting function. Also the option to download all the set of gene-associated stoichiometric reactions for a specific organism from the KEGG database is available.

### 2.1. Introduction

Genome-scale metabolic network reconstructions (GMNR) specify the chemical reactions catalyzed by hundreds of enzymes (registered in enzyme commission – E.C.) and cover the molecular function of a substantial fraction of a genome [21]. The main goal of these network reconstructions is to relate the genome of a given organism with its physiology, incorporating every metabolic transformation that this organism can perform [22, 23]. The GMNR are converted into computational models for simulation of metabolism and gain insight into

the complex interactions that give rise to the metabolic capabilities [24, 25]. The predictive accuracy of a model depends on the comprehensiveness and biochemical fidelity of the reconstruction [26].

The GMNR construction process can be synthesized into two fundamental stages: (1) draft network reconstruction, here the reactions associated with the enzymes that participate in the metabolism of a particular organism, are downloaded from specialized genome, biochemical and metabolic databases; and (2) refinement of the network manually or using computational algorithms. Similar steps are performed during the construction of a tissue-specific metabolic reconstruction, defined as a subset of reactions included in a genome-scale metabolic reconstruction that are highly associated with the metabolism of a specific tissue[27]. They are constructed from measured gene expression or proteomic data and permit characterize or predict the metabolic behavior of a tissue under any physiological condition. Due to only the reactions associated with an enzyme or gene can be mapped from the measured data, the spontaneous reactions, and non-facilitated transport reactions are missing in first stages of a tissue-specific reconstruction.

The refinement stage of the reconstruction is a process to restore the connectivity network, where network gaps in the draft reconstruction are identified, and candidate reactions to fill the gap are find in literature and databases [5, 28]. Since the network reconstructions typically involve thousands of metabolic reactions, the refinement of them can be a very complex task [23].The network gaps can be associated with dead-end metabolites which cannot be imported/produced by any of the reactions in the network; or metabolites that are not used as substrates or released by any of the reactions in the network. When the metabolic network is transformed into a metabolic steady-state model to optimize the distribution of metabolic flux under an objective function, the presence of this type of metabolites can be problematic, due to the flux cannot pass through them due to the incomplete connectivity with the rest of the network [28].

In a high-quality model, all reactions should be able to carry flux if all relevant exchange reactions are available [23]. The lack of flux in dead-end metabolites is propagated downstream/upstream, depending if the metabolites are not produced or not consumed, giving rise to additional metabolites that cannot carry any flux [28]. This can block out the net flux of the objective function and when it is not blocked, bias in the biological conclusions increase. The manual refinement is an iterative process to assemble a higher confidence compendium of organism-specific metabolism in a draft metabolic network reconstruction [29–31]. This type refinement requires time and a labor intensive of use of available literature, databases and experimental data [31, 32]. Given GMNR account for hundreds or thousands of biochemical reactions, this task is very complex and can lead to both, the introduction of new errors and to overlook some others.

Network gap refinement also can be performed using implemented algorithms to identify blocked reactions through the optimization of the metabolic model, and to fill metabolic functions gaps, by means of the search of reactions associated with the missing functions in available databases [23, 26]. However, this approach only fill gaps for a single biological objective function; other implemented approaches based on the identification and filling of dead-ends metabolites included in **GAMS** [28] and **COBRA** [33] packages, allows fill the networks gaps for all metabolism, generating a list of reactions that permit restore the network connectivity and eliminate blocked reactions that no carry flux in a model steady state [31]. However, implemented **COBRA** [33] gap fill algorithm operate as tools under the commercial MATLAB<sup>®</sup> environment and **GAMS** [28] only allow the gap fill using MetaCyc databases as reference [34].

With the aim of offering an open source tool that facilitates the refinement and depuration of drafts network reconstructions and metabolic models, we introduce the **g2f** R package. It includes five functions to identify and fill gaps, as well as, to calculate the addition cost of a reaction, depurate metabolic networks of blocked reactions (no activated under any scenario) and a function to associate GPR to reactions included in a metabolic reconstruction using the KEGG database as a reference.

## 2.2. Installation and functions

The **g2f** package includes four functions and is available for download and installation from CRAN, the Comprehensive R Archive Network. To install and load it, just type:

```
> install.packages("g2f")  
> library(g2f)
```

The **g2f** package requires R version 2.10 or higher. Development releases of the package are available on the GitHub repository <http://github.com/gibbslab/g2f>.

### Downloading a reference from the KEGG database

The KEGG database is a collection of databases widely used as a reference in genomics, metagenomics, metabolomics and other omics studies, as well as for modeling and simulation in systems biology [35]. At today, the database includes genomes, biological pathways and its associated stoichiometric reactions for 346 eukaryotes, 3947 bacteria, and 238 archaea. The **getReference** function download from the KEGG database all the KO-associated stoichiometric reactions, and their correspondent E.C. numbers for a customized organism (using KEGG organism ID). Based in the KOs associated to a reaction, their respective GPR is construed as follows: All gene associated to a determined KO are linked by an **AND** operator,

413 after that, when a reaction has more than one KO associated, previously linked genes are  
 414 now joined by an OR operator. As an example, to download all (1392 reactions) stoichiometric  
 415 reactions associated to *Escherichia coli* just type:

```
> E.coli <- getReference(organism = "eco")
```

## 416 Calculating the addition cost

The reactions mapping based on metabolites and posterior addition is a very basic solution for gap fill process which increases the number of dead-end metabolites. As a way to reduce the addition of new dead-end metabolites, the `additionCost` function calculates based on metabolites that constituted the new reaction and those that constitute the stoichiometric reactions present in the metabolic reconstruction a cost (in terms of new metabolites) to be added following the equation 2-1.

$$additionCost = \frac{n(\text{metabolites}(\text{newReaction}) \notin \text{metabolites}(\text{reactionList}))}{n(\text{metabolites}(\text{newReaction}))} \quad (2-1)$$

417 As an example, we select a sample of reactions from the downloaded reference for *E. coli*  
 418 and calculate the addition cost for the remaining reactions (6 first values are showed).

```
> reactionList <- sample(E.coli$reaction,10)
> head(
+   additionCost(reaction = E.coli$reaction,
+               reference = reactionList)
+ )
```

```
[1] 0.4000000 0.4000000 0.4000000 0.4000000 0.3333333 0.3333333
```

## 419 Performing a gap find and fill

420 To identify network gaps in a metabolic network and fill it from a reference, the `gapFill`  
 421 function perform internally several steps: (1) The dead-end metabolites are identified from  
 422 the stoichiometric matrix using functions included in the `minval` package, (2) the candidate  
 423 reactions to be added are identified by metabolite mapping, (3) the addition cost of each  
 424 candidate reaction is calculated, (4) the candidate reactions with an addition cost lower or  
 425 equal that the user-defined limit are added to the reaction list and finally (5) process return  
 426 to step 1 until no more original-gaps can be filled under the user-defined limit. Function  
 427 returns a set of candidate stoichiometric reactions to fill the original-gaps included in the  
 428 metabolic network.

429 As an example, we show how to fill the dead-end metabolites included in the previously  
 430 selected sample using all downloaded stoichiometric reactions from the KEGG database for  
 431 *E. coli* as the reference.

```

> gapFill(reactionList = reactionList,
+         reference = E.coli$reaction,
+         limit = 1/4
+ )

23 Orphan reactants found
12 Orphan reactants found
11 Orphan reactants found
11 Orphan products found
[1] "D-Mannonate + NAD+ <=> D-Fructuronate + NADH + H+"
[2] "ATP + Thymidine <=> ADP + dTMP"
[3] "dTTP + H2O <=> dTMP + Diphosphate"
[4] "(R,R)-Tartaric acid + NAD+ <=> 2-Hydroxy-3-oxosuccinate + NADH + H+"
[5] "Hypoxanthine + NAD+ + H2O <=> Xanthine + NADH + H+"
[6] "Ammonia + 3 NAD+ + 2 H2O <=> Nitrite + 3 NADH + 3 H+"
[7] "L-Glutamine + H2O <=> L-Glutamate + Ammonia"
[8] "ATP + Deamino-NAD+ + Ammonia <=> AMP + Diphosphate + NAD+"
[9] "Ammonia + NAD+ + H2O <=> Hydroxylamine + NADH + H+"
[10] "ATP + Glutathione + Spermidine <=> ADP + Orthophosphate + Glutathionylspermidine"
[11] "2 Glutathione + NAD+ <=> Glutathione disulfide + NADH + H+"
[12] "Glutathione + H2O <=> Cys-Gly + L-Glutamate"
[13] "L-Proline + NAD+ <=> (S)-1-Pyrroline-5-carboxylate + NADH + H+"
[14] "L-Glutamate 5-semialdehyde + NAD+ + H2O <=> L-Glutamate + NADH + H+"
[15] "ATP + Pantothenate <=> ADP + D-4'-Phosphopantothenate"

```

## 432 Identifying blocked reactions

433 To identify the blocked reactions included in a metabolic model, the `blockedReactions`  
 434 function set each one of the reactions included in the model (one by time) as the objective  
 435 function and optimize through Flux Balance Analysis the model. Reactions that not parti-  
 436 cipate in any possible solution during all evaluations are returned as a blocked reaction.

437  
 438 As an example, we identify the blocked reactions in the *E. coli* core metabolic model included  
 439 in the 'sybil' package.

```

> data("Ec_core")
> blockedReactions(Ec_core)

|=====| 100%
[1] "EX_fru(e)" "EX_fum(e)" "EX_mal_L(e)" "FUMt2_2" "MALt2_2"

```

## 440 2.3. Summary

441 We introduced the `g2f` package to find the dead-end metabolites included in a metabolic  
 442 reconstruction, and fill it from the stoichiometric reactions of a reference, filtering candidate

443 reactions using a weighting function and a user-defined limit. We show step by step the  
444 functionality of each procedure included in the package using a reference downloaded from  
445 the KEGG database for *E. coli*, and the core metabolic model included in the ‘sybil’  
446 package.



# 3. Constraining a tissue-specific metabolic reconstruction: Incorporating expression data as FBA limits through ‘exp2flux’ R package.

**Original title:** exp2flux: Convert Gene EXPression Data to FBA FLUXes.

**Written by:** *Daniel Osorio, Kelly Botero, Janneth Gonzalez and Andrés Pinzón-Velasco*

## Abstract

Computational simulations of metabolism can help to predict the metabolic phenotype of an organism in response to different stimuli, through constraint-based modeling approaches. To recreate specific metabolic phenotypes and enhance the model predictive accuracy, several methods for the integration of transcriptomics data into constraint-based models have been proposed. The majority of available implemented methods are based on the discretization of data to incorporate constraints into the metabolic models via boolean logic representation, which reduces the accurate of physiological representations. The implemented methods for gene-expression data integration as continuous values are very few. The **exp2flux** package was designed as a tool to incorporate in a continuous way the gene-expression data as FBA flux limit in a metabolic reconstruction. Also, a function to calculate the differences between fluxes in different metabolic scenarios was included.

## 3.1. Introduction

Metabolism is a cellular system suited for developing studies at the systemic level of the genotype-phenotype relationship and genetic interactions [21]. Genome sequencing projects have been contributed to our understanding of the metabolic capabilities in cellular systems since functional annotation of the gene products (enzymes) allow the reconstruction of genome-scale metabolic networks (GMNs), that summarize these metabolic capabilities consistently and compactly in a stoichiometric matrix [36, 37]. The GMNs can be converted

471 into computational models to predict the metabolic phenotype of an organism in response  
472 to different stimuli, through constraint-based modeling approaches [33, 38]. A widely used  
473 approach to perform *in silico* metabolic simulations is the flux balance analysis (FBA), a  
474 linear optimization method which uses the imposed mass balance and constraints (that re-  
475 present genetic or environmental conditions) to define the space of feasible steady-state flux  
476 distributions of the network and then identify optimal network states that maximize a defi-  
477 ned objective function [21, 39].

478  
479 Given that steady-state simulations assume enzymatic constant rate and does not consider  
480 the real expression of each gene or the subcellular localization of gene products, flux con-  
481 straints based on different “omics” data (such as transcriptomics, proteomics, and metabolo-  
482 mics) must be integrated into the GMNs, in order to recreate specific metabolic phenotypes  
483 and enhance the model predictive accuracy [22, 40–42]. Integrative methods have a power-  
484 ful potential to describe molecular and biochemical mechanisms of organisms metabolism  
485 under specific environmental or genetic conditions, as well as, to allow contextualize high-  
486 throughput data [43].

487  
488 Several algorithms and methods have been developed to integrate experimental data at  
489 GMNs [44]. Given the increase of gene expression data, these algorithms have focused on  
490 incorporate transcriptomics data into constraint-based models [45–47], and in this way con-  
491 strain the flux distribution (solution space) in GMNs [33, 48]. Each of the algorithms is based  
492 on the assumption that mRNA transcript levels are a strong indicator of the level of protein  
493 activity [49]. The algorithms differ mainly in the way to integrate expression data, some of  
494 the implemented algorithms incorporate data in a discrete or continuous way, using absolute  
495 values for a single condition, or the relative expression levels between different conditions  
496 [50]. The most of the methods are based on the discretization of data to incorporate con-  
497 straints into the metabolic models via boolean logic representation (activation/inactivation  
498 flux) [49]. Even when continuous integration could be more accurate for physiological repre-  
499 sentation of the continuous of the reactions activity gradient [42], the methods of continuous  
500 integration are very few [50].

501  
502 With the aim of offer an open source tool that facilitates the integration of gene-expression  
503 data as a continuous constraint (FBA limits) in metabolic models, we introduce the ‘exp2flux’  
504 R package. The exp2flux package incorporates a previously described but not implemented  
505 continuous gene-expression data integration method [51, 52]. The implemented method is  
506 based on the association of ‘omics’ data to the genes included in the Gene-Protein-Reaction  
507 (GPR) related to each reaction in a genome-scale metabolic model each reaction [5] and  
508 considers different possible biological scenarios that occur during the catalysis of biochemical  
509 reactions [51]. Also, a function to calculate the difference in reaction fluxes between simulated  
510 scenarios was included.

## 3.2. Installation and functions

`exp2flux` includes two functions and is available for download and installation from CRAN, the Comprehensive R Archive Network. To install and load it, just type:

```
> install.packages("exp2flux")
> library(exp2flux)
```

The `exp2flux` package requires R version 2.10 or higher. Development releases of the package are available on the GitHub repository <http://github.com/gibbslab/exp2flux>.

### Inputs

Functions included in `exp2flux` package takes as input two kind of objects, metabolic models as an object of class `modelorg` for the ‘`sybil`’ R package, and gene-expression data as an object of class `ExpressionSet`, a container for high-throughput assays and experimental metadata described in the ‘`Biobase`’ Bioconductor package.

### Converting gene expression data to FBA limits

Gene-protein-reaction (GPR) associations indicate which gene has what function into a genome-scale metabolic network and are represented as boolean relationships between genes. This function calculates and assigns the flux boundaries for each reaction based in their associated GPR. Value is obtained as follows: (1) When two genes are associated with an AND operator according to the GPR rule, a minimum function is applied to their associated expression values. In the AND case, down-regulated genes alter the reaction acting as the enzyme formation limiting, due both are required to the enzymatic complex formation. In turn, (2) when the genes are associated with an OR rule, each one of them can code an entire enzyme to act as a reaction catalyst. In this case, a sum function is applied for their associated expression values. To missing gene expression values, the function do a data imputation and assigns one of: ‘`min`’, ‘`1q`’, ‘`mean`’, ‘`median`’, ‘`3q`’, or ‘`max`’ expression value calculated from the genes associated to the same metabolic pathway. Metabolic pathway assignation for each reaction is performed through an organism-specific search in the KEGG database. In the case of not possible pathway assignment to a gene, the value to be assigned is calculated from all gene expression values. The fluxes boundaries of exchange reactions are not modified. To show the potential use of data integration through the `exp2flux` function, in this example, we simulate values to represent gene-expression data and integrate it into the *Escherichia coli* core metabolic model included in the ‘`sybil`’ R package.

Integration of gene-expression data begins loading the ‘`exp2flux`’, ‘`sybil`’ and ‘`biobase`’ required packages.

```
> library(exp2flux)
> library(sybil)
> library(Biobase)
```

543 After that, the *E. coli* core metabolic model can be loaded from the ‘sybil’ package. The  
 544 model includes 95 biochemical reactions associated with 137 genes in 69 GPR rules.

```
> data("Ec_core")
```

545 Five different measures to represent the gene-expression data for each gene included in the  
 546 metabolic model was simulated, generated matrix was converted to an **ExpressionSet**.

```
> geneExpression <- ExpressionSet(assayData = matrix(
+   data = runif(n = 5*length(Ec_core@allGenes), min = 0, max = 1000),
+   nrow = length(Ec_core@allGenes),
+   dimnames = list(c(Ec_core@allGenes))
+ ))
> geneExpression
```

```
ExpressionSet (storageMode: lockedEnvironment)
assayData: 137 features, 5 samples
  element names: exprs
protocolData: none
phenoData: none
featureData: none
experimentData: use 'experimentData(object)'
Annotation:
```

547 Incorporation of gene-expression data into the metabolic model through **exp2flux** function  
 548 requires two objects, a metabolic model and an **ExpressionSet** as arguments. The **exp2flux**  
 549 function returns a constrained metabolic model.

```
> mEc_core <- exp2flux(
+   model = Ec_core,
+   expression = geneExpression
+ )
> mEc_core
```

```
model name:          Ecoli_core_model
number of compartments 2
                     C_c
                     C_e
number of reactions:  95
number of metabolites: 72
number of unique genes: 137
objective function:   +1 Biomass_Ecoli_core_w_GAM
```

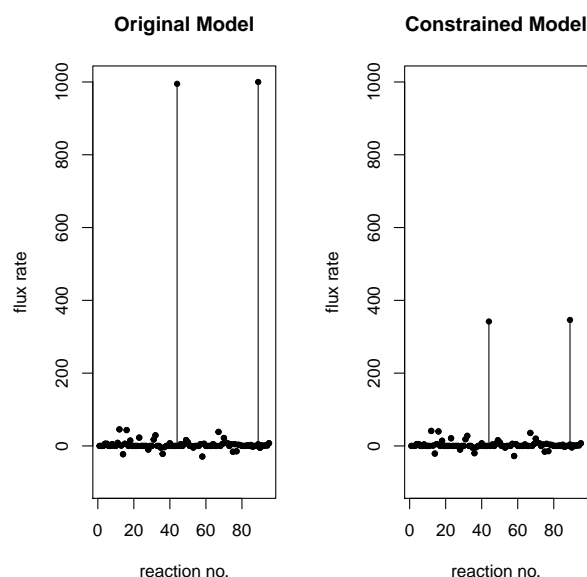
550 To visualize the metabolic changes induced by the incorporation of gene-expression data as  
 551 FBA limits of the reactions included in the metabolic model, a Flux Variability Analysis was  
 552 performed using the original *E. coli* core metabolic model and the constrained one.

```
> par(mfcol=c(1,2))
> plot(fluxVar(Ec_core),main="Original Model",ylim=c(-100,1000))

|           :           |           :           | 100 %
|=====| :~)

> plot(fluxVar(mEc_core),main="Modified Model",ylim=c(-100,1000))

|           :           |           :           | 100 %
|=====| :~)
```



**Figure 3-1.:** Flux differences between an unconstrained and a constrained model. Constraints were calculated through the `exp2flux` R package using simulated gene expression data.

## 553 Identifying flux changes between scenarios

The measurement of flux change for each reaction between metabolic scenarios is a task generally carried out manually and oriented directly to the research objective. However, at system level analysis this process can become laborious. The `fluxDifferences` function calculates the fold change for each common reaction between metabolic scenarios. *Fold change* is a measure that describes how much a quantity changes going from an initial to a final value. Implemented algorithm in the `fluxDifferences` functions are described in equation 4-1, function takes as argument two valid models for the ‘`sybil`’ R package and a customizable

threshold value to filter functions to be reported.

$$foldChange : \mathbb{R} \times \mathbb{R} \rightarrow \mathbb{R}$$

$$(rFluxModel1, rFluxModel2) \mapsto \begin{cases} rFluxModel2, & rFluxModel1 = 0; \\ \frac{(rFluxModel2 - rFluxModel1)}{|rFluxModel1|}, & \text{Other cases} \end{cases} \quad (3-1)$$

554 As an example, we report the fold change of all reactions with an absolute change greatest  
555 or equal to 2-fold between the unconstrained and constrained metabolic scenarios simulated  
556 previously.

```
> fluxDifferences(  
+   model1 = Ec_core,  
+   model2 = mEc_core,  
+   foldReport = 2  
+ )
```

	fluxModel1	fluxModel2	foldChange
ADK1	4.547474e-13	1.000444e-11	21
D_LACt2	-6.821210e-13	1.364242e-12	3
LDH_D	-6.821210e-13	1.364242e-12	3

### 557 3.3. Summary

558 We introduced the **exp2flux** package, an implementation of a previously described method  
559 to integrate gene-expression data in a continuous way into genome-scale metabolic network  
560 reconstructions. We show as an example, how the integration of a set of simulated data  
561 modifies the behavior of the *E. coli* core metabolic model using Flux Balance Analysis  
562 simulations. Also, a example of the measurement of flux change between metabolic scenarios  
563 was showed.

## 4. Exploring the neuroprotective effects of tibolone during astrocytic metabolic inflammation: a flux balance analysis approach

**Written by:** *Daniel Osorio, Janneth Gonzalez and Andrés Pinzón-Velasco*

### **Abstract:**

Inflammation is a complex biological response to injuries, metabolic disorders or infections and its dysregulation induces many complex diseases through astrocytic dysfunction. The increase of free saturated fatty acid produce a metabolic inflammation response, generally associated with the induction of diverse intracellular stresses, such as mitochondrial oxidative stress, endoplasmic reticulum stress, and autophagy defects. Astrocytes respond to inflammation through a complex reaction called astrogliosis. During astrogliosis, glial cells generally associated with several beneficial activities in the CNS, also act as a source of inflammatory mediators and as generators of ROS that have the potential to damage neurons. In search of compounds with neuroprotective effects that imitate the neuroprotective actions of steroids without their prejudicial side effects; the synthetic neurosteroid Tibolone was identified. Although Tibolone has been shown to exert neuroprotective actions in cultured and under ischemia injury rat neurons, its specific actions on glial cells have received very little attention. Nevertheless, is not well know the effects of tibolone on glial cells that allow its neuroprotective action. In this work, we model and simulate the metabolic inflammation response in mature astrocytes through Flux Balance Analysis (FBA), and explore the neuroprotective effects of tibolone under the inflamed state. We focused on identification of changes in metabolic pathways activation, functional products, gliotransmitter release and the neuroprotective effects mediated by tibolone over inflamed scenario. The generated network consisted of 1262 genes encoding for enzymes performing 2747 reactions distributed across eight compartments, which was studied using a constrained-based modeling approach to recreating three different scenarios in mature astrocytes (healthy, inflamed and medicated), and validated with available experimental evidence. From our analysis, we predict that

tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes.

## 4.1. Introduction

### Astrocyte-Neuron Metabolic Relationships

Astrocytes are the most abundant cells in the human brain and play important roles in the central nervous system (CNS) [53]. They are highly associated with several homeostatic functions such as glutamate, ion, and water homeostasis, energy storage in the form of glycogen, synapse formation, and remodeling, defense against oxidative stress, scar formation, tissue repair and modulation of synaptic activity via the release of gliotransmitters [54]. Astrocytes metabolize glucose in the anaerobic way to produce lactate, which is released to neurons through monocarboxylate transporters [55]. Lactate is used in neurons as an energy substrate after its conversion to pyruvate and subsequently to ATP via oxidative phosphorylation [56].

Astrocytes play an important role in glutamate-mediated synaptic activity [57]; according to the astrocyte–neuron lactate shuttle model, astrocytes respond to glutamate-induced activation by increasing their rate of glucose uptake and the release of lactate into the extracellular space, increasing the lactate available to be used by neurons to supply their energetic needs [58]. Glutamate is uptake by astrocytes through the glutamate-aspartate transporter and glial glutamate transporter-1, inducing events that involve the activation of  $\text{Na}^+/\text{K}^+$ -ATPase and maintaining extracellular glutamate at homeostatic levels [59]. Part of incorporated glutamate is converted to glutamine through glutamine synthetase, which is only associated with glial cells and released to neurons using electroneutral systems-N transporters coupled to  $\text{Na}^+$  and  $\text{H}^+$  [60]. In neurons, glutaminase enzyme converts glutamine back into glutamate which can be used again for neurotransmission or metabolized into the neuronal Krebs cycle [61].

Astrocytes uptake and release many other substances related to synaptic transmission [62]. However D-serine, a neurotransmitter that acts as a co-agonist with glutamate at NMDA receptors is one of the most important [57]. Due in the brain, only glial cells can synthesize serine, all available D-serine at synapsis is associated to be primarily produced and secreted by astrocytes [60]. D-serine is synthesized in astrocytes by serine racemase from L-serine [63]. Serine and glycine are involved in a cycle between astrocytes and neurons similar to the glutamate-glutamine cycle [64]. Additionally, to these energetic and synaptic support associated functions, astrocytes also play an important role in the reduced glutathione (GSH) metabolism of the brain [65]. GSH is the major cellular antioxidant and plays an important neuroprotective role [66]. Cellular GSH levels are closely correlated with cell survival under adverse conditions [67]; it is synthesized from glutamate, cysteine, and glycine and releases



directly from astrocytes through GSH transporters ion-independent in a concentration gradient dependent transport [68].

This strong metabolic cooperation between astrocytes and neurons allows predicting that even a small astrocytic dysfunction might cause and/or contribute neurodegenerative processes [69]. Homeostatic astrocyte function is required for neuronal survival after different brain insults, such as inflammation, glucose deprivation, traumatic brain injury and ischemia [66, 70]. Astrocytes protect neurons of the most important factors that contribute to neuronal cell death such as glutamate-mediated excitotoxicity leading to disturbances in calcium and sodium intracellular metabolism, mitochondrial dysfunction, oxidative stress, cytokines and toxins [53, 54, 59, 71].

## Astrocytes response to Inflammation

Inflammation is a complex biological response to injuries, metabolic disorders or infections, and its dysregulation induces many complex diseases through astrocytic dysfunction [66, 72, 73]. In the brain, inflammatory response acts as a defense mechanism against any threat to homeostatic state inducing changes in glucose metabolism and release of pro-inflammatory factors [67]. Inflammation responses in CNS are mediated by glial cells that acquire reactive phenotypes to participate in repair mechanisms [53, 66, 74].

Astrocytes, as glial cells are highly sensitive cells to inflammatory mediators, they respond to inflammation through a complex reaction named astrogliosis [75]. During astrogliosis, glial cells generally associated with several beneficial activities in the CNS, also act as a source of inflammatory mediators and as generators of reactive oxidant species (ROS) that have the potential to damage neurons [76]. Astrogliosis is characterized by a low regulation of mitochondrial dynamics that result in mitochondrial failure [77]. Mitochondrial failure induces the deregulation of  $\text{Ca}^{2+}$  homeostasis and increased ROS generation, both of which are linked to neurotoxicity [54]. At metabolic level, inflammatory process has been associated with an increase of free saturated fatty acid in comparison with healthy conditions in some brain tissues [78].

The increase of free saturated fatty acid induce metabolic inflammation, a response associated with the induction of diverse intracellular stresses, such as mitochondrial oxidative stress, endoplasmic reticulum stress, and autophagy defects [66]. Lipid excess in metabolic inflammation activates  $\text{IKK}\beta$  and  $\text{NF-}\kappa\beta$  signaling pathways, which ultimately impairs leptin and insulin hormonal signaling and further triggers the synthesis and release of increased amounts of ROS and pro-inflammatory cytokines ( $\text{TNF-}\alpha$  and IL-6) from glial cells to sustain the neuroinflammatory state [79]. Enhanced ROS generation by reactive glial cells trigger mitochondria dysfunction in neuron, which induces neuronal apoptosis, the prerequisite for

a diverse number of neurodegenerative conditions [80].

## In silico Systems Biology and Inflammation

Inflammatory pathways are evolutionarily conserved, complex, redundant and interconnected [81]. These characteristics difficult each attempt to understand any disease having inflammation as its core using the traditional reductionism-based scientific method and the current regulatory framework [82]. Traditional methods generally focus on single molecules and genes as the targets of study and potential therapy development, nevertheless, mechanistic simulation through a translational systems biology methods allows lead to an understanding of the origin of patterns based on ‘omic’ data integration in order to facilitate the design of novel therapies [83].

Inflammation is a complex system, which is characterized by sensitivity to initial conditions, positive and negative feedback loops, combined robustness and fragility, and the emergence of nonintuitive behaviors [84]. Translational Systems Biology to inflammation is focused on simulated clinical trials, trying to progress toward personalized diagnostics, personalized medicine, and the rational design of drugs [81].

## Tibolone

Drugs as steroids compounds are the most potent and effective agents in controlling chronic inflammatory diseases [85]. However, steroids prescription is limited due to their adverse side effects [86]. Some steroids synthesized in the nervous system, called ‘neurosteroids’, display beneficial neuroprotective properties, which may be of particular importance in the treatment of diseases where inflammation and neurodegeneration is predominant including age-dependent dementia, stroke, epilepsy, spinal cord injury, Alzheimer’s disease (AD) and Parkinson’s disease (PD) [87].

Neuroprotective actions of molecules that may imitate the neuroprotective actions of steroids without the prejudicial side effects, such as selective estrogen receptor modulators (SERMs) and selective tissue estrogenic activity regulators (STEARs) have been tested in previous studies [88, 89]. Tibolone is a compound, traditionally used as hormone replacement therapy in post-menopausal women [90], that has been shown neuroprotective effects in cultured and under ischemia injury rat neurons [91].

Tibolone is a synthetic steroid drug with estrogenic, progestogenic, and weak androgenic actions; is metabolized in three compounds, two major active metabolites, 3 $\alpha$ -hydroxy tibolone and 3 $\beta$ -hydroxy tibolone acting as potent agonists of the estrogen receptor (ER) and its metabolite  $\Delta$ 4tibolone acting as agonists of the progesterone and androgen receptors

[92]. Tibolone and their metabolites have tissue selective action mechanisms (progestogenic, androgenic and estrogenic) reported in liver, bone, breast and brain according to receptor interaction and activation [88]. Nevertheless, actually, is not well know the effects of tibolone on glial cells that allow its neuroprotective effects [70]. Previous studies have shown that 3-hydroxy-metabolites of tibolone exert agonistic actions on human astrocytes through the activation of estrogen receptors, indicating that astrocytes are a target for tibolone [91].

In this work, we simulate the metabolic inflammatory response in mature astrocytes caused by the increased uptake of palmitate, the most common free saturated fatty acid. We model and simulate the metabolic response using a translational system biology approach called Flux Balance Analysis (FBA) described in methods. We focused on the identification of changes in metabolic pathways activation, functional products, gliotransmitter release and the neuroprotective effects mediated by tibolone in the inflamed scenario.

## 4.2. Material and Methods

### Tissue Specific Model Construction

The tissue specific model construction process started with the identification of all enzyme-coding genes expressed over the mean in at least 50 % of samples for healthy human astrocytes indexed in the GEO database [93] as GSE73721 [94]. Gene identifier conversion from GeneCards[95] to ENTREZ [96] was performed through ‘UniProt.ws’ R Package [97]. Reactions associated with the identified genes were mapped from the Human Genome-Scale Metabolic Reconstruction RECON 2.04 downloaded from the VMH Lab (<https://vmh.uni.lu>) [98]. The R package ‘g2f’ [99] was used to identify and fill the gaps using all no gene-associated reactions included in RECON 2.04, as well as to identify and remove all blocked reactions from the reconstruction.

All reactions involved in the conversion of extracellular glutamate, glycine, cysteine and glucose to extracellular glutamine, glycine, serine-D, reduced glutathione, lactate, and ATP respectively were added. Exchange reactions were limited to components of the Dulbecco’s Modified Eagle Medium (DMEM) as input and gliotransmitters (glutamine, D-serine, ATP, glutamate), reduced glutathione, lactate, glucose, nitric oxide, prostaglandins and leukotrienes as output. Finally, syntax, mass-charge validation and creation of SBML files were carried out through the ‘minval’ R Package [100]. Reaction limits (upper and lower bounds) were constrained proportionally to the mean gene expression reported for genes included in Gene-Protein-Reaction (GPR) [5] associated to each reaction in samples of 47 to 63 years old using the ‘exp2flux’ R package [101]. All Flux Balance Analysis (FBA) were performed using the ‘sybil’ [102] R Package running under R 3.3.1 [103].

## Flux Balance Analysis

FBA is a linear optimization method for simulating metabolism that allows identifying the set of reactions involved in the production of a biological response within a metabolic model [104]. The metabolic reactions are represented internally as a stoichiometric matrix ( $S$ ), of size  $m \times n$ , where  $m$  represents the compounds and  $n$  the reactions; the entries in the matrix are the stoichiometric coefficients of the metabolites participating in a reaction [105]. The flux through all of the reactions in a network is represented by the vector  $v$ , which has a length of  $n$ . The concentrations of all metabolites are represented by the vector  $x$ , with length  $m$ . The systems of mass balance equations at steady state,  $\frac{dx}{dt} = 0$  or  $S \times v = 0$ . FBA seeks to maximize or minimize an objective function which can be any linear combination fluxes, to obtain a flux for each reaction, indicating how much each reaction contributes to the objective function [104].

**Table 4-1.:** Main metabolic capabilities associated to astrocytes represented as the set of objective functions used to evaluate neuroprotective effects of Tibolone under inflamed scenarios

ID	FORMULA REACTION	DESCRIPTION
Glu2Gln	1 glu_L[e] $\Rightarrow$ 1 gln_L[e]	Glutamate - Glutamine Cycle
Gly2SerD	1 gly[e] $\Rightarrow$ 1 ser_D[e]	Glycine to D-serine conversion
Glc2Lac	1 glc_D[e] $\Rightarrow$ 2 lac_L[e]	Lactate production from Glucose
Glc2ATP	1 glc_D[e] $\Rightarrow$ 36 atp[e]	ATP production from Glucose
Cys2GTHRD	1 cys_L[e] + 1 glu_L[c] + 1 gly[c] $\Rightarrow$ 1 gthrd[e]	Catch of Cysteine to produce reduced Glutathione

FBA for healthy, inflamed and medicated scenarios was resolved using GLPK 4.60, setting the generic human biomass reaction included in RECON 2.04 and each one of reactions described in table 4-1 as objective functions. Models were analyzed by comparing fluxes between scenarios, metabolites production rate and a sensitivity analysis.

## Metabolic Scenarios

To test neuroprotective effects of tibolone during astrocytic metabolic inflammation we define three different metabolic scenarios. A ‘healthy’ scenario, where palmitate uptake rate was freely set by optimizer; an ‘inflamed’ scenario, where uptake rate of palmitate was forced to be stable in the mean of the half maximal inhibitory concentration (IC50) value for all objective functions included in table 4-1. IC50 values were calculated through a robustness analysis performed using uptake of palmitate (‘EX\_hdca(e)’ in RECON 2.04) as control

reaction and 1000 points in the range from 0 to 1 mMgDW<sup>-1</sup>h<sup>-1</sup> for each objective function. Uptake value where each objective function reached IC50 was selected and subsequently averaged. Finally, a medicated scenario, defined as an inflamed scenario that includes 279 reactions associated with estradiol-derived compounds and ten specific reactions associated with Tibolone action mechanism not included in RECON 2.04 described in table 4-2.

**Table 4-2.:** Set of reactions added to recreate the medicated scenario model over the astrocyte tissue-specific model. Reactions are the representation of tibolone metabolism in the brain following the reported by Kloosterboer (2004).

ID	FORMULA REACTION	DESCRIPTION
T1	tibolone[e] $\Leftrightarrow$	Tibolone exchange reaction
T2	tibolone[e] $\Leftrightarrow$ a3Ohtibolone[e]	3 $\alpha$ hydroxytibolone interconversion
T3	tibolone[e] $\Leftrightarrow$ b3Ohtibolone[e]	3 $\beta$ hydroxytibolone interconversion
T4	tibolone[e] $\Rightarrow$ d4tibolone[e]	$\Delta$ 4tibolone isomer formation
T5	b3Ohtibolone[e] $\Rightarrow$ d4tibolone[e]	$\Delta$ 4tibolone isomer formation from 3 $\beta$ -hydroxytibolone
T6	a3Ohtibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3 $\alpha$ -hydroxytibolone
T7	b3Ohtibolone[e] $\Rightarrow$ estradiol[c]	Estradiol receptor agonist action mechanism of 3 $\beta$ -hydroxytibolone
T8	d4tibolone[e] $\Rightarrow$ prgstrn[c] + tststerone[c]	Progesterone and androgen receptor activation by tibolone $\Delta^4$ isomer
T9	a3Ohtibolone[e] $\Leftrightarrow$ a3SOtibolone[e]	3 $\alpha$ hydroxytibolone interconversion to sulfated inactive compounds
T10	a3SOtibolone[e] $\Rightarrow$	Tibolone inactive form in blood

## Metabolic Changes

Metabolic changes across metabolic scenarios were measured through two different approximations. Flux differences for each reaction between optimized scenarios were measured using the fold change calculated as described in equation 4-1.

$$foldChange = \frac{valueModel2 - valueModel1}{|valueModel1|} \quad (4-1)$$

## 768 Pro-inflammatory, Anti-inflammatory and Tibolone Action Mechanism 769 Associated Enzymes

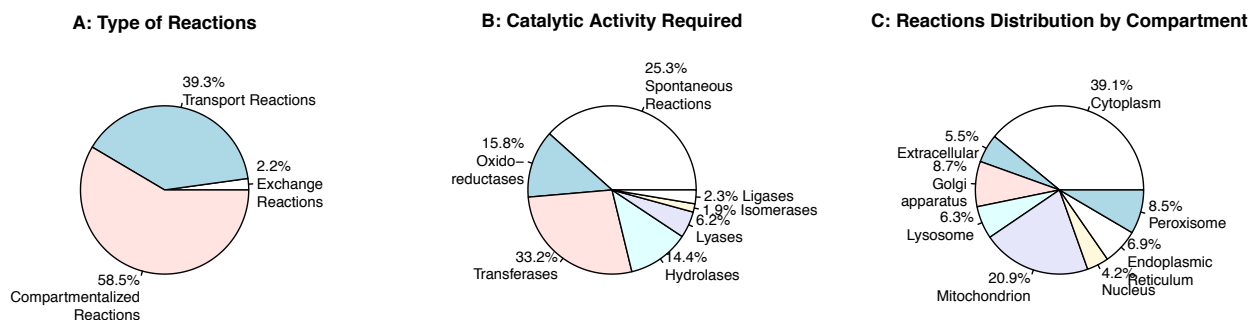
770 Enzymes involved in pro-inflammatory and anti-inflammatory responses as well as in the tibo-  
771 lone action mechanism were identified through sensitivity analysis as follows: Pro-inflammatory  
772 enzymes, are those that catalyze reactions that being knocked out allows an increase of ob-  
773 jective function value. Anti-inflammatory enzymes are those associated with reactions that  
774 have a fold-change greatest equal to 2, and at being knocked out reduce even more the objec-  
775 tive function value. Tibolone action mechanism associated enzymes are those that catalyze  
776 reactions that being knocked out inhibit entirely the metabolic effect of tibolone.

## 777 4.3. Results

778 All data, code, software and output files used in the developing of this work, are available  
779 to be downloaded from GitHub URL: <https://github.com/dosorio/masterThesis> as a free  
780 repository.

## 781 Tissue Specific Metabolic Model

782 Generated astrocyte tissue-specific model describe the metabolism of 1956 compounds in a  
783 total of 2747 biochemical reactions associated to 1262 unique genes. Biochemical reactions  
784 include 60 exchange and 1080 transport reactions (79 % gene associated, facilitated or active  
transport) as is shown in figure 4-1A. To describe the astrocyte tissue-specific metabolic



**Figure 4-1.:** Distribution of biochemical reactions included in the astrocyte tissue-specific metabolic model, classification based in **A:** Type of reaction, **B:** Catalytic activity required and **C:** Associated compartment.

785 model, reactions were classified on the basis of required enzymatic activity to be catalyzed  
786 according to their Enzyme Commission (E.C) numbers (Fig. 4-1B), sub-cellular locations  
787

according to metabolites compartment (Fig. 4-1C), and metabolic pathways assigned in the KEGG database (Fig. 4-2). Based on the associated enzyme to each biochemical reaction 33.2 % of them are catalyzed by a transferase enzyme, 15.8 % by an oxidoreductase, 14.4 % by a hydrolase, 6.2 % by a lyase, 2.3 % by a ligase, 1.9 % by an isomerase enzyme and 25.3 % of them are spontaneous reactions without enzyme or gene associated. In the classification shown in figure 4-1C, the cytosolic and mitochondrial reactions contributed to 60 % of the total reactions in the model. The other 40 % of reactions are distributed in six other compartments as follows: 8.7 % occurs in Golgi apparatus, 8.5 % in the peroxisome, 6.9 % in the endoplasmic reticulum, 6.3 % in the lysosome, 4.2 % in nucleus, finally 5.5 % of them occurs outside the cell, in the extracellular space. Reactions included in astrocyte model are associated with 113 metabolic pathways reported in the KEGG database [6]. Almost 50 % reactions are associated to 10 main metabolic pathways, highly related to astrocytes metabolism and neuron support metabolic functions [58, 64, 106–109]. Entirely distribution of reactions in metabolic pathways is shown in figure 4-2.

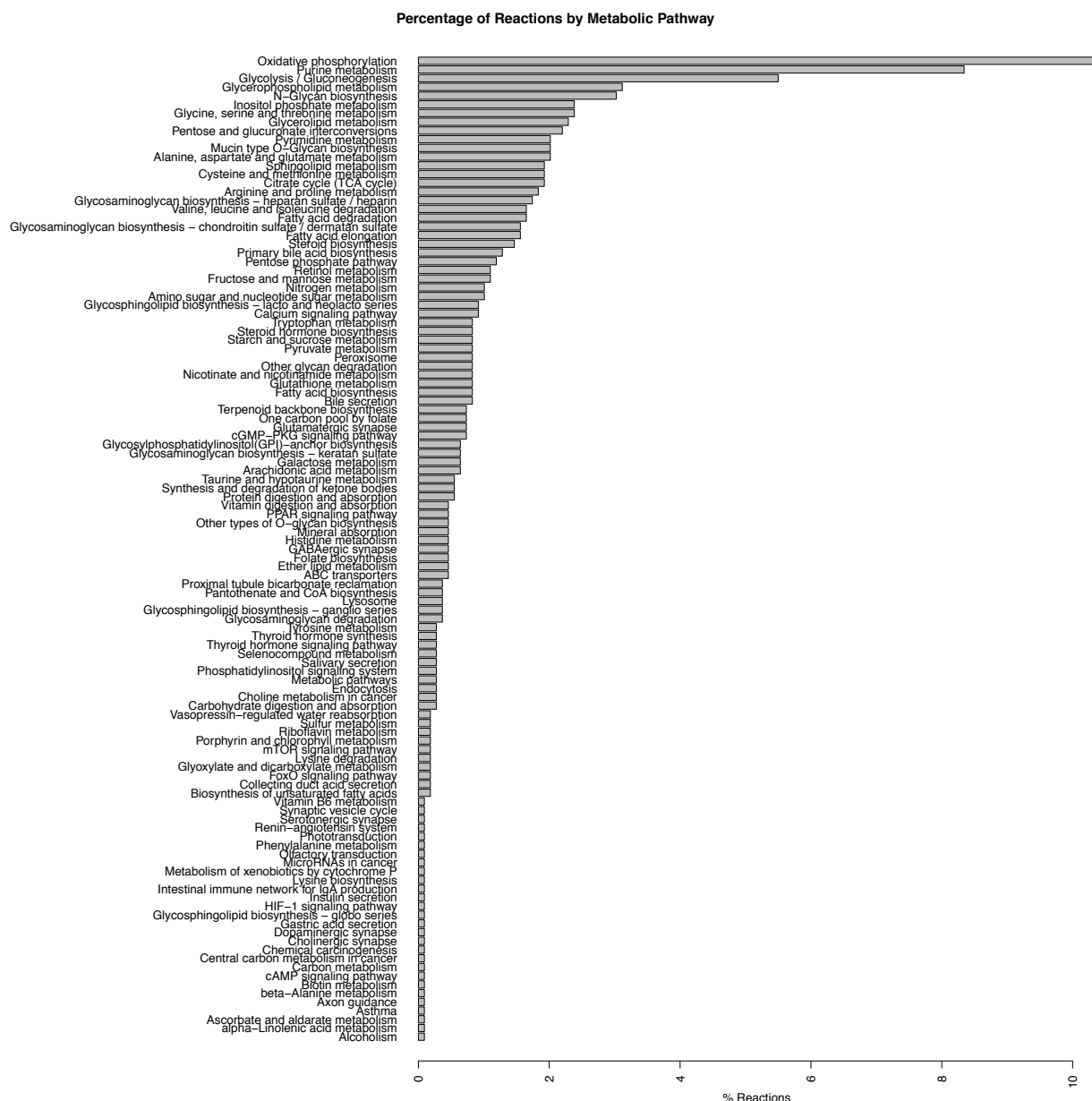
## Healthy Scenario

As previously reported by the Das *et al.* wet lab, healthy human astrocytes grow up in DMEM culture medium [110]. Our metabolic simulation allows to predict an astrocytes slow grow rate ( $0.37 \text{ mMgWD}^{-1}\text{h}^{-1}$ ) under DMEM medium.

In our healthy scenario (Fig. 4-3), astrocytes activate the 52 % of model reactions and prefer a glucose-based metabolism, equal than found by Çakir *et al.* and Bhowmick *et al.* in resting conditions [64, 111]. Glucose is catabolized and constitutively released by astrocytes as lactate without any stimuli [112]. This observation is highly expected due astrocytes release large amounts of lactate in the extracellular space which can be used by neurons to supply their energy needs [67]. In our simulations, other gliotransmitters are synthesized and released by astrocytes only under specific stimulus (objective functions) and their release rate was used as a reference to the comparison between scenarios (Fig. 4-6).

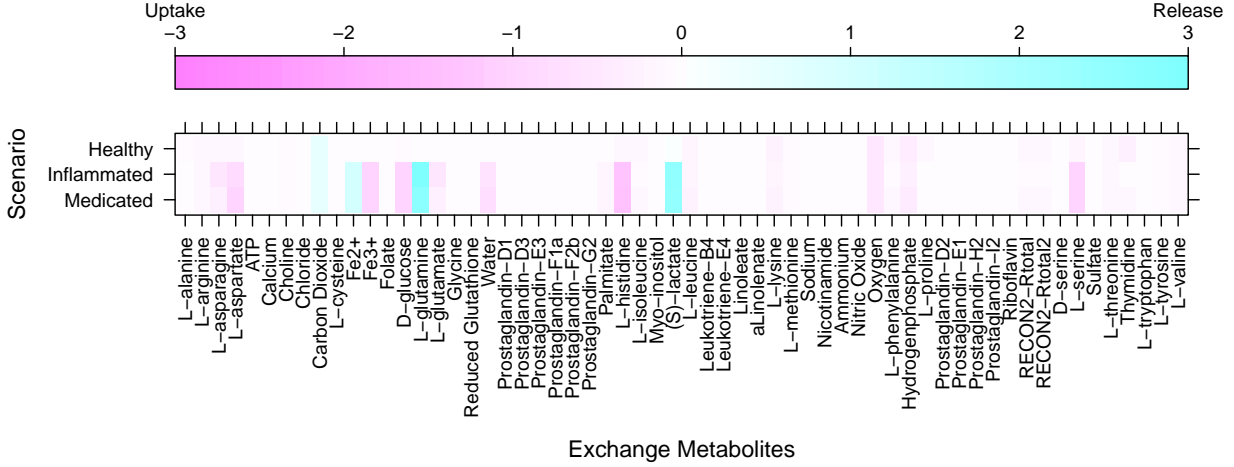
## Inflamed Scenario

In the model, the healthy scenario was perturbed to generate the inflamed scenario. The palmitate-induced IC50 value calculated for a set of metabolic functions (Table 4-1) was  $0.208 \pm 0.024 \text{ mMgDW}^{-1}\text{h}^{-1}$ . Calculated IC50 value is the same (0.2 mM) used by Liu *et al.* in wet lab to induce an astrogliosis reaction [113]. Inflamed scenario increases the demand for L-asparagine, L-aspartate, iron, D-glucose, L-glutamate, histidine, L-serine and the release of L-glutamine and lactate (Fig. 4-3). This response is typical of astrocytes in astrogliosis where neuroinflammation lead homeostatic disturbances [114], such as iron accumulation, within CNS cells [66]. Iron accumulation has been demonstrated in several neurodegenerative diseases as AD, and PD, where it has been postulated to promote disease



**Figure 4-2.:** Pathways associated with biochemical reactions included in the astrocyte tissue-specific metabolic model. Pathway association was assigned based in the categorization of the KEGG database.





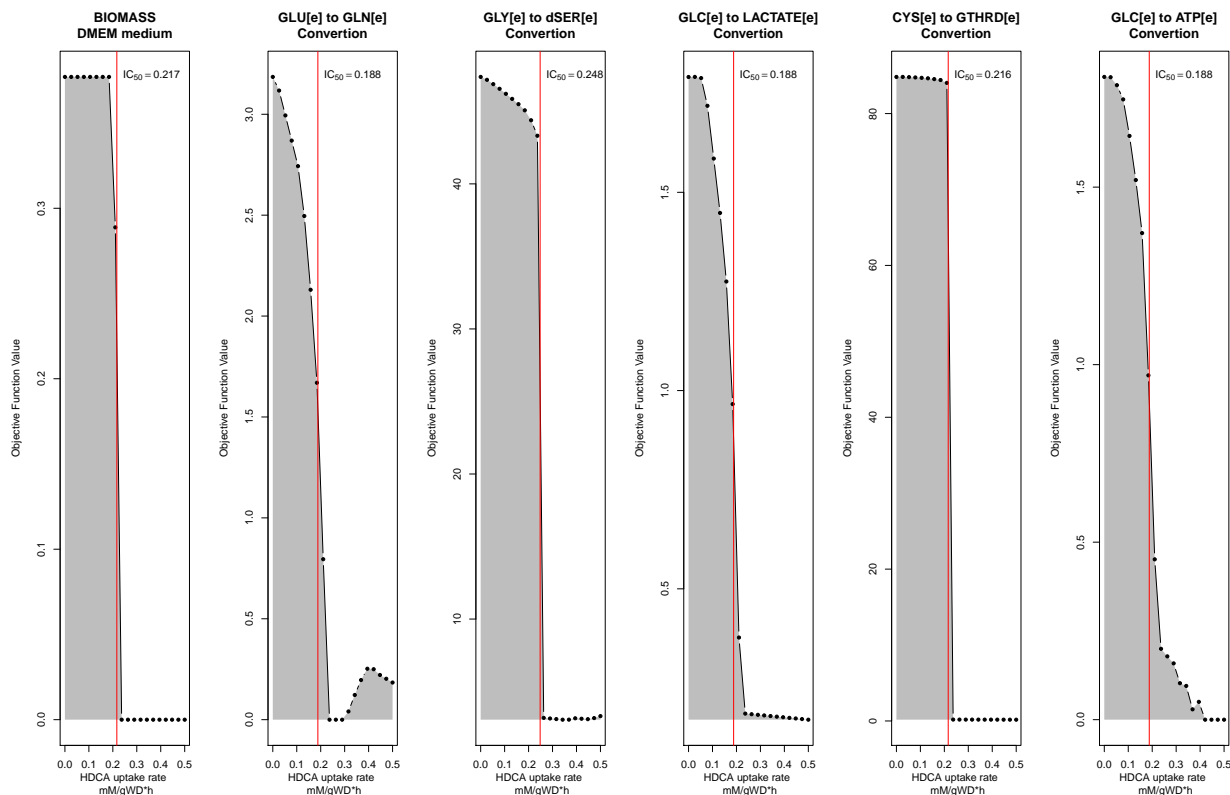
**Figure 4-3.:** The exchange rate of metabolites between metabolic scenarios using the generic biomass reaction included in RECON 2.04 as the objective function

by augmenting microglial pro-inflammatory activity, altering mitochondrial function, and inducing ROS production [115].

Inflammation although induce the neuronal release of glutamate that may result in the recruitment of neurons in the neuroinflammatory process [116]. Glutamate uptake into astrocytes disinhibits glycolytic enzymes that result in glucose uptake; this glucose is generally processed glycolytically, leading to the synthesis and release of lactate [66]. As neurons cannot generate glutamine from glutamate owing to the lack of the glutamine synthetase enzyme, uptake glutamate is returned to neurons via synaptic clefts in the form of glutamine [117].

Histidine uptake increase was previously reported and suggested as a biomarker of metabolic inflammation [118]; it acts as a free-radicals scavenger and could reduce the levels of IL-6, TNF- $\alpha$ , CRP and inhibit the  $H_2O_2$  and TNF- $\alpha$  induced by IL-8 secretion [119, 120]. Aspartate, present in the brain as N-Acetyl-L-aspartate (NAA) is synthesized and stored in the neurons but is hydrolyzed in glial cells [121]. NAA act as an anti-proliferation, antiangiogenic, and anti-inflammatory molecule through the decrease of the amount of prostaglandin E2 (PGE2) in astroglial cells [122]. L-Asparagine, in turn, acts as a regulator of ammonia toxicity through the increase of  $Na^+$  intracellular concentration when is co-transported inside astrocytes [123]; asparagine induce a  $Ca^{2+}$  response comparable to GABA-induced  $Ca^{2+}$  transients in a dose-dependent manner [124].

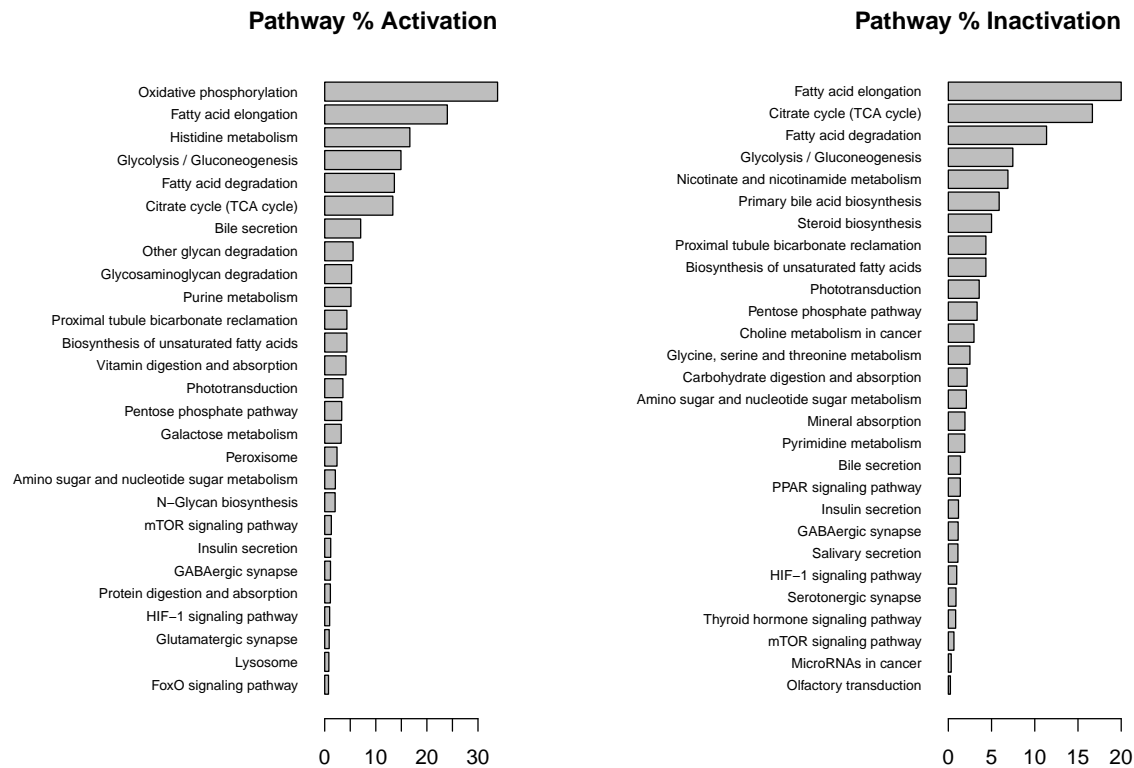
L-serine and L-asparagine uptake increase may be related to the cell survival process that



**Figure 4-4.:** Robustness analysis to calculate palmitate-induced IC50 value for each objective function described in table 4-1. The red line represents the calculated IC50 value.

switches cellular metabolism to be highly dependent of nonessential amino acids available in extracellular space such as glutamine, serine, glycine, arginine, and asparagine [125]. Moreover, under the inflamed scenario, our astrocyte model release a very small amount of prostaglandin D2. The release of this prostaglandin was previously associated to induce the depolarization and potentiate the actions of simultaneously applied transmitters such as GABA, taurine, glutamate, and aspartate in astrocytes [126].

In our inflamed scenario, astrocytes activate the 46.6 % of model reactions (5.6 % less than healthy scenario) and affect the biomass flux rate of 586 reactions in comparison with the healthy scenario. Main metabolic changes occur in the activation of oxidative phosphorylation, histidine metabolism, and fatty acid degradation pathways; as well as an inactivation of TCA and glycolysis pathways (Fig. 4-5). Inflammation affects in a negative way all metabolic objective functions evaluated except the release of D-serine. In comparison to the healthy scenario, growth rate over DMEM medium decrease in a 15.6 %, the catch of cysteine to produce reduced glutathione in a 59.3 %, conversion of glucose to ATP in a 72 %, and to



**Figure 4-5.:** Metabolic pathways affected by metabolic inflammation. Percentage of activation and inactivation was calculated in comparison with genes associated with each pathway in the KEGG database

lactate in a 74.4 %; finally, conversion of extracellular glutamate in glutamine reduces in a 67.7 % (Fig. 4-6).

Based on performed sensibility analysis, we identify two pro-inflammatory reactions candidate to be knocked out (Table 4-3), that when being blocked increases the value of the objective function above the maximum value set (in an 11.45 % and 5.14 % respectively) for the inflamed scenario. Reactions are associated to the formimidoyl-transferase cyclodeaminase (FTCD) enzyme and the Aquaporin-8, a water transport protein.

FTCD enzyme, previously reported as over-expressed in high-fat diets [127], contribute with one-carbon units from histidine degradation to the folate pool [128]. In turn, the Aquaporin 8, generally associated to ammonia and water transport [129], has been proposed as a biomarker for inflammation processes where was in contrary way to our observations, highly correlated to cellular defence against severe oxidative stress [130].

**Table 4-3.:** Set of reactions with pro-inflammatory potential identified through a sensibility analysis over inflamed scenario.

ID	REACTION DESCRIPTION	H. FLUX	I. FLUX	FOLD CHANGE
FTCD	Formimidoyltransferase cyclodeaminase	0.39	1.28	2.28
H2Otm	H2O transport mitochondrial	-0.26	2.44	10.44

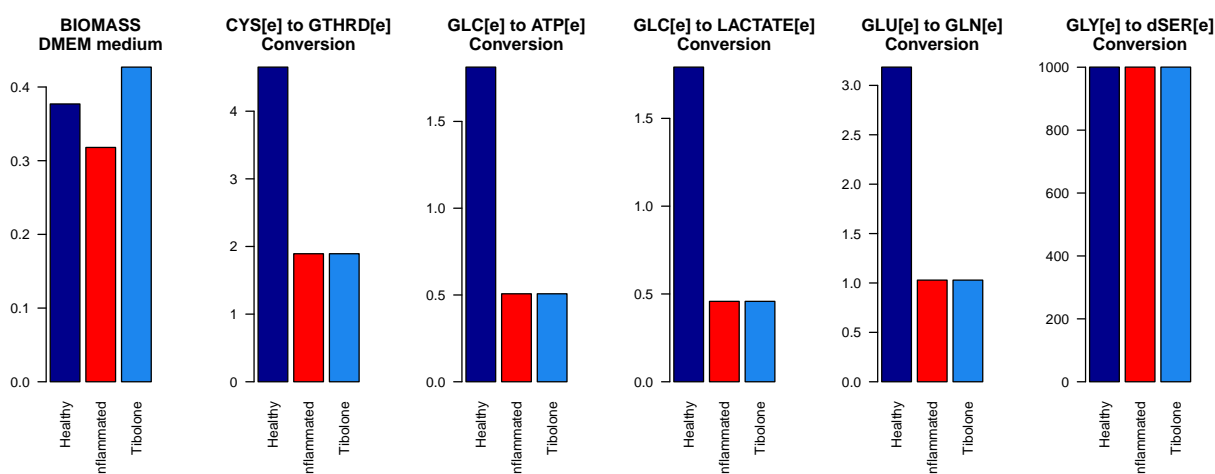
**Table 4-4.:** Set of reactions with anti-inflammatory potential identified through a sensibility analysis over inflamed scenario.

ID	REACTION DESCRIPTION	H. FLUX	I. FLUX	FOLD CHANGE
AKGMALtm	$\alpha$ -ketoglutarate/malate transporter	-0.17	-1.3	-6.85
NADH2_u10m	NADH dehydrogenase mitochondrial	0.12	0.37	2.17
r0639	Lauroyl-CoA: acetyl-CoA C-acyltransferase.	0.02	0.09	4.04
r0653	cMyristoyl-CoA: acetyl-CoA C-myristoyl transferase	0.02	0.09	4.04
r0714	(S)-3-Hydroxyhexadecanoyl-CoA: NAD <sup>+</sup> oxidoreductase	0.02	0.09	4.04
r0716	(S)-3-Hydroxyhexadecanoyl-CoA hydrolyase	0.02	0.09	4.04
r0718	(S)-3-Hydroxytetradecanoyl-CoA: NAD <sup>+</sup> oxidoreductase	0.02	0.09	4.04
r0720	(S)-3-Hydroxytetradecanoyl-CoA hydrolyase	0.02	0.09	4.04

As well as pro-inflammatory reactions, eight anti-inflammatory reactions were identified. Identified reactions (Table 4-4) have a change between scenarios greatest equal to 2-fold and when being blocked decreased, even more, the value of the objective function in comparison with the healthy scenario. The majority of identified reactions (r0639, r0653, r0714, r0716, r0718 and r0720) are involved in the fatty acid elongation in mitochondria through acyl-CoA association [131]. The elongation system, is responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain, and play an important role in the maintenance of membrane lipid composition as well as in the generation of precursors for cell signaling molecules (such as eicosanoids and sphingosine-1 phosphate), energy production, and other unknown pathways involving with cancer growth. [132].

## Medicated Scenario

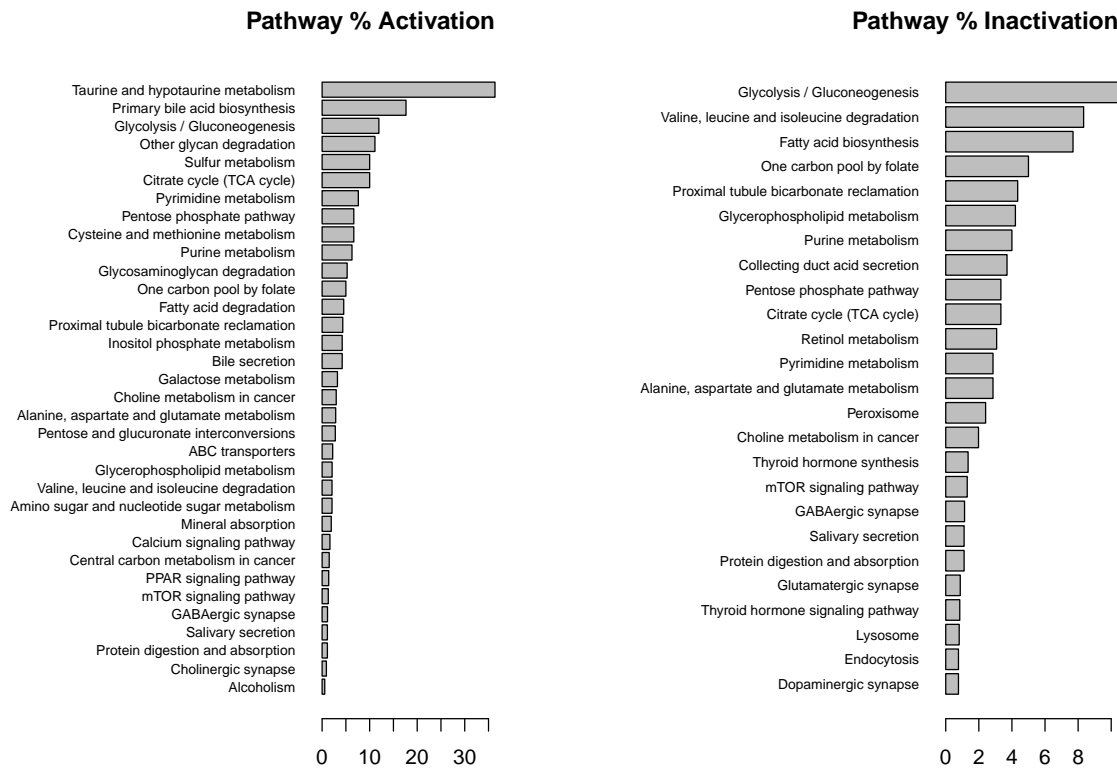
The inflamed scenario with the 279 reactions associated with tibolone and estradiol-derived compounds metabolism, defined as our medicated scenario displays several neuroprotective effects. Medicated scenario increase the demand of L-aspartate and in turn decreases the demand for L-asparagine, L-glutamate and the release of L-glutamine in comparison to the inflamed scenario (Fig. 4-3).



**Figure 4-6.:** The response of the main astrocytes metabolic capabilities to different modeled scenarios.

The reduction of L-glutamate and L-glutamine uptake/release rate mediated by Tibolone effects could be associated with a neuroprotective effect through a reduction of neurotoxicity mediated by L-glutamate in astrocytes [62]. L-glutamate is a contributing factor in neuronal damage induced by inflammation, traumatic brain injury, stroke, and in most of the chronic neurodegenerative diseases, such as PD and AD [133].

In our medicated scenario, astrocytes activate 46.6 % of model reactions, equal than in inflamed scenario. Nevertheless, tibolone effects affect the biomass flux rate of 948 reactions in comparison with the inflamed scenario; main metabolic changes occurs through the activation of several metabolic pathways with neuroprotective actions associated such as taurine metabolism, which has been shown to be tissue-protective in many models of oxidant-induced injury [134], gluconeogenesis which is accelerated and facilitate the conversion of fatty acids into ketone bodies under steroid-mediated effects [135], calcium and PPAR signaling pathways (Fig. 4-7).



**Figure 4-7.:** Metabolic pathways affected by tibolone effects over inflamed scenario. Activation and inactivation percentage was measured in comparison with genes associated to each pathway in the KEGG database

Tibolone does not show directly effect over the main associated neuron-supportive capabilities (Table 4-1) by affected by inflammation, however, it shows a high related activity as a potentiator of growth rate (Fig. 4-6). An increase of growth rate (13.26 % higher than in healthy scenario) could be associated with an increase in cell viability or as an increase in the proliferative potential in cells [136]. Due that proliferative potential was not evidenced in the inflamed scenario, this proliferative potential must not be associated with astrogliosis mediated proliferation in astrocytes [53]. However, it could be associated with the proliferative side effect of steroid compounds as was previously reported in other cell types where tibolone was tested in wet lab [137, 138].

Based on performed sensibility analysis over the 289 reactions associated with tibolone and estradiol-derived compounds metabolism, we identify a set of four reactions that when being individually knocked out, block entirely the tibolone effects (Table 4-2). Identified reactions are catalyzed by an alcohol dehydrogenase (E.C. 1.1.1.1) and a Cytochrome P450 associated with the PPAR signaling pathway. Both enzymes were previously reported with

**Table 4-5.:** Set of reactions associated with tibolone required to execute its neuroprotective effects. Reactions were identified through a sensibility analysis over the medicated scenario.

ID	REACTION DESCRIPTION	GENES IN ASTROCYTE DATA
r0739	Alcohol Dehydrogenase	ADH4, ADH5, ADH7
r2518	ATP-binding Cassette (ABC)	ABCD3
RE1804M	Cholestanetriol 26-monooxygenase	CYP27A1
RE1807M	Cholestanetriol 26-monooxygenase	CYP27A1

ROS reduction through redox reactions mediated by alcohol dehydrogenase (ADH) and posterior release associated to a cytochrome P450 [139, 140].

## 4.4. Conclusion

In this work, a tissue-specific metabolic network for mature astrocyte has been developed, and three different scenarios were modeled. Modeled scenarios allowed identify the metabolic changes between a healthy and an inflamed scenario as well as from an inflamed to a tibolone medicated scenario. The model was capable of yielding results which were in correspondence to the experimentally proved metabolic processes [110, 113]. From our study, adverse effects associated with the increase of palmitate uptake were described based on exchange, metabolite production, and metabolic pathways perturbed under inflammatory response. Sensibility analysis performed through constrained-based modeling approach and FBA methods permitted recognize two possible reactions and their associated enzymes susceptible to be knocked out to reduce inflammatory processes.

Based on literature reports we modeled a tibolone medicated scenario used to identify and describe the neuroprotective effects of this synthetic neurosteroid under an inflamed scenario in astrocytes; our main results suggest that tibolone execute their neuroprotective effects through a reduction of neurotoxicity mediated by L-glutamate in astrocytes. L-glutamate [62]. We also found a tibolone associated increase in growth rate probably in concordance to previously reported side effects of steroids in other human cell types [137, 138]. Identified enzyme associated reactions with tibolone effects and their action mechanisms are highly consistent with reported previously by our associated wet lab [70, 141].

## A. MINVAL algorithms

---

### MINVAL algorithm 1: isValidSyntax

---

**input** : A set of stoichiometric reaction with the following format: H2O[c] + Urea-1-carboxylate[c] <=> 2 CO2[c] + 2 NH3[c]. Where arrows and plus signs are surrounded by a "space character". It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form => or <=>. Meaning that arrows like ==>, <==>, -> or -> will not be parsed and will lead to errors.

**output**: A boolean value TRUE or FALSE for each stoichiometric reaction

```

1  foreach stoichiometric reaction do
2      create an empty boolean vector V;
3      if each metabolite has only just one coefficient then
4          | add TRUE to V
5      else
6          | add FALSE to V
7      if metabolites coefficients are not surrounded by parentheses then
8          | add TRUE to V
9      else
10         | add FALSE to V
11     if arrow symbol is between blank spaces then
12         | add TRUE to V
13     else
14         | add FALSE to V
15     if arrow symbol is <=> or => then
16         | add TRUE to V
17     else
18         | add FALSE to V
19     if metabolites names are separated by a plus symbol (+) between blank spaces then
20         | add TRUE to V
21     else
22         | add FALSE to V
23     if substituents position are joined by an hyphen to the metabolite name then
24         | add TRUE to V
25     else
26         | add FALSE to V
27     if all elements of V are TRUE then
28         | return TRUE
29     else
30         | return FALSE

```

---



---

**MINVAL algorithm 2: isBalanced**


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**input :**

reactionList: A set of stoichiometric reaction with the following format:  $\text{H}_2\text{O}[\text{c}] + \text{Urea-1-carboxylate}[\text{c}] \rightleftharpoons 2 \text{CO}_2[\text{c}] + 2 \text{NH}_3[\text{c}]$ . Where arrows and plus signs are surrounded by a "space character". It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form  $\Rightarrow$  or  $\Leftarrow$ . Meaning that arrows like  $\Rightarrow$ ,  $\Leftarrow$ ,  $\rightarrow$  or  $\leftarrow$  will not be parsed and will lead to errors.

referenceData: A chemical table containing data to evaluate the balance

ids: A mandatory id of metabolites id column in the referenceData

mFormula: An optional id of molecular formula column in the referenceData

mWeight: An optional id of molecular weight column in the referenceData

mCharge: An optional id of net charge column in the referenceData

**output:** A boolean value TRUE or FALSE for each stoichiometric reaction

```

1  foreach stoichiometric reaction do
2      extract reactants ;                                /* Applying regular expressions */
3      extract reactants coefficients ;                    /* Applying regular expressions */
4      multiply reactants by coefficients;
5      map reactants in referenceData using the given id;
6      if all reactants were mapped then
7          if mFormula is given then
8              split molecular formulas and sum all atoms ;    /* Applying regular expressions */
9          else
10             if mWeight is given then
11                 sum all molecular weights
12             else
13                 if mCharge is given then
14                     sum all molecular charges
15                 else
16                     return NA
17     else
18         return NA
19     extract products ;                                /* Applying regular expressions */
20     extract products coefficients ;                    /* Applying regular expressions */
21     multiply products by coefficients;
22     map products in referenceData using the given id;
23     if all products were mapped then
24         if mFormula is given then
25             split molecular formulas and sum all atoms ;    /* Applying regular expressions */
26         else
27             if mWeight is given then
28                 sum all molecular weights
29             else
30                 if mCharge is given then
31                     sum all molecular charges
32                 else
33                     return NA
34     else
35         return NA
36 if sum of reactants is equal to sum of products then
37     return TRUE
38 else
39     return FALSE

```

---

---

**MINVAL algorithm 3: orphanReactants**


---

**input** : A set of stoichiometric reaction with the following format:  $\text{H}_2\text{O}[\text{c}] + \text{Urea-1-carboxylate}[\text{c}] \rightleftharpoons 2 \text{CO}_2[\text{c}] + 2 \text{NH}_3[\text{c}]$ . Where arrows and plus signs are surrounded by a “space character”. It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form  $\Rightarrow$  or  $\Leftarrow$ . Meaning that arrows like  $\Rightarrow$ ,  $\Leftarrow$ ,  $\rightarrow$  or  $\leftarrow$  will not be parsed and will lead to errors.

**output**: A set of metabolites that are not produced in any other reaction or just are involved in just one reaction.

```

1 create three empty vectors  $m$ ,  $r$  and  $p$ ;
2 foreach stoichiometric reaction do
3   | split stoichiometric reaction by arrow ( $\rightleftharpoons$ ) and plus symbol (+);
4   | remove stoichiometric coefficients;
5   | remove all blank spaces;
6   | add metabolites to  $m$ ;
7 compute the absolute frequency for each metabolite;
8 remove from  $m$  vector all metabolites with frequencies greater or equal than 2;
9 for all irreversible reactions do
10  |  $r \leftarrow$  extract reactants ;                               /* Applying regular expressions */
11  |  $p \leftarrow$  extract products ;                             /* Applying regular expressions */
12 return elements of  $((r \not\subset p) \cup m)$ 

```

---



---

**MINVAL algorithm 4: orphanProducts**


---

**input** : A set of stoichiometric reaction with the following format:  $\text{H}_2\text{O}[\text{c}] + \text{Urea-1-carboxylate}[\text{c}] \rightleftharpoons 2 \text{CO}_2[\text{c}] + 2 \text{NH}_3[\text{c}]$ . Where arrows and plus signs are surrounded by a “space character”. It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form  $\Rightarrow$  or  $\Leftarrow$ . Meaning that arrows like  $\Rightarrow$ ,  $\Leftarrow$ ,  $\rightarrow$  or  $\leftarrow$  will not be parsed and will lead to errors.

**output**: A set of metabolites that are not produced in any other reaction or just are involved in just one reaction.

```

1 create three empty vectors  $m$ ,  $r$  and  $p$ ;
2 foreach stoichiometric reaction do
3   | split stoichiometric reaction by arrow ( $\rightleftharpoons$ ) and plus symbol (+);
4   | remove stoichiometric coefficients;
5   | remove all blank spaces;
6   | add metabolites to  $m$ ;
7 compute the absolute frequency for each metabolite;
8 remove from  $m$  vector all metabolites with frequencies greater or equal than 2;
9 for all irreversible reactions do
10  |  $r \leftarrow$  extract reactants ;                               /* Applying regular expressions */
11  |  $p \leftarrow$  extract products ;                             /* Applying regular expressions */
12 return elements of  $((p \not\subset r) \cup m)$ 

```

---

## B. G2F algorithms

---

### G2F algorithm 1: additionCost

---

**input :**

reaction: A stoichiometric reaction with the following format:  $\text{H2O}[\text{c}] + \text{Urea-1-carboxylate}[\text{c}] \rightleftharpoons 2 \text{CO2}[\text{c}] + 2 \text{NH3}[\text{c}]$ .

Where arrows and plus signs are surrounded by a "space character". It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form  $\Rightarrow$  or  $\Leftarrow$ . Meaning that arrows like  $\Rightarrow$ ,  $\Leftarrow$ ,  $\rightarrow$  or  $\rightarrow$  will not be parsed and will lead to errors.

reference: A set of stoichiometric reactions with the same format of reaction

**output:** The addition cost of a stoichiometric reaction based in the metabolites that compound a reference

```

1 refMet ← extract all metabolites from reference ;           /* Applying regular expressions */
2 rxnMet ← extract all metabolites from reaction ;           /* Applying regular expressions */
3 return  $\frac{(| \text{rxnMet} | - | \text{rxnMet} \in \text{refMet} |)}{| \text{rxnMet} |}$ 

```

---



---

### G2F algorithm 2: blockedReactions

---

**input :** A valid model for the 'sybil' R package. An object of class `modelorg`.

**output:** A set of ID's associated to reactions without flux under all scenarios.

```

1 create a empty vector F;
2 foreach reaction in the model do
3   set all objective coefficients as 0;
4   assign selected reaction as objective function;
5   optimize the model;
6   identify reactions with flux different to 0;
7   add IDs of identified reactions to F;
8 return model reaction IDs  $\notin$  F

```

---

---

**G2F algorithm 3: gapFill**


---

**input :**

**reactionList:** A set of stoichiometric reactions with the following format:  $\text{H2O}[c] + \text{Urea-1-carboxylate}[c] \rightleftharpoons 2 \text{CO2}[c] + 2 \text{NH3}[c]$ . Where arrows and plus signs are surrounded by a "space character". It is also expected that stoichiometry coefficients are surrounded by spaces. It also expects arrows to be in the form  $\Rightarrow$  or  $\Leftarrow$ . Meaning that arrows like  $\Rightarrow$ ,  $\Leftarrow$ ,  $\rightarrow$  or  $\leftarrow$  will not be parsed and will lead to errors.

**reference:** A set of stoichiometric reaction with the same format of **reactionList**

**limit:** An addition cost value to be used as a limit to select reactions to be added. Is calculated as  $\frac{\text{NumberNewMetabolites}}{\text{NumeOfMetabolites}}$  for each reaction.

**woCompartment:** A boolean value TRUE to define if compartment labels should be removed of the **reactionList** stoichiometric reactions, FALSE is used as default.

**consensus:** A boolean value TRUE to define if **reactionList** and **newReactions** should be reported as a unique vector or FALSE if just **newReactions** should be reported.

**output:** A set of stoichiometric reactions that fill the model gaps.

```

1 if woCompartment is TRUE then
2   remove compartments of reactionList and reference metabolites ;      /* Applying regular expressions */
3 orphanOriginal  $\leftarrow$  orphanReactants(reactionList)  $\cup$  orphanProducts(reactionList);
4 to.add  $\leftarrow$  reactionList;
5 do
6   Compute additionCost(reference);
7   Select stoichiometric reactions with additionCost(reference)  $\leq$  limit;
8   Extract orphanReactants(reactionList);
9   Compute |orphanReactants(reactionList)  $\cap$  orphanOriginal|;
10  Add stoichiometric reaction that include the orphanReactants(reactionList) within selected stoichiometric reactions
    with additionCost(reference)  $\leq$  limit to to.add array;
11 while |orphanOriginal  $\cap$  orphanReactants (reactionList)|  $\geq$  |orphanOriginal  $\cap$  orphanReactants(reactionList  $\cup$ 
    to.add)|;
12 newReactions  $\leftarrow$  unique(to.add);
13 do
14   Compute additionCost(reference);
15   Select stoichiometric reactions with additionCost(reference)  $\leq$  limit;
16   Extract orphanProducts(reactionList);
17   Compute |orphanProducts(reactionList)  $\cap$  orphanOriginal|;
18   Add stoichiometric reaction that include the orphanProducts(reactionList) within selected stoichiometric reactions
    with additionCost(reference)  $\leq$  limit to to.add array;
19 while |orphanOriginal  $\cap$  orphanProducts (reactionList)|  $\geq$  |orphanOriginal  $\cap$  orphanProducts(unique(reactionList
     $\cup$  to.add))|;
20 newReactions  $\leftarrow$  unique(to.add  $\cup$  newReactions)  $\setminus$  reactionList;
21 if consensus is TRUE then
22   return reactionList  $\cup$  newReactions
23 else
24   return newReactions

```

---

## C. EXP2FLUX algorithms

---

### EXP2FLUX algorithm 1: fluxDifferences

---

**input :**

model1: A valid model for the 'sybil' package.

model2: A valid model for the 'sybil' package. Must have the same reactions (reaction number and reaction identifiers) as "model1" with different restrictions.

foldReport: A threshold value to be reported. All reactions with a greater or equal fold change than the given threshold are reported.

**output:** The calculated fold change for the fluxes of two given metabolic models.

```

1 fluxModel1 ← extract fluxDistribution(optimize(model1));
2 fluxModel2 ← extract fluxDistribution(optimize(model2));
3 foreach reaction ∈ (model1 ∩ model2) do
4   if fluxModel1reaction is equal to 0 then
5     return fluxModel2reaction
6   else
7     if (1-(fluxModel2reaction/fluxModel1reaction) ≤ foldReport) then
8       return 1-(fluxModel2reaction/fluxModel1reaction)

```

---

---

**EXP2FLUX algorithm 2: exp2flux**


---

**input :**

model: A valid model for the 'sybil' package.

expression: A valid ExpressionSet object (one by treatment).

organism: A valid organism identifier for the KEGG database. List of valid organism identifiers are available in:  
<http://rest.kegg.jp/list/organism>.

typeID: A string to define the type of ID used in GPR's. One of entrez or kegg must be given

missing: A character string specifying the value to be used in missing cases; must be one of min, 1q, mean, median, 3q, or max.

scale: A boolean value to specify if data must be scaled to assign a value of 1000 as max.

**output:**

```

1  if organism and typeID is given then
2  |   Download from the KEGG database the metabolic pathways associated to each gene
3  foreach non exchange reaction ∈ model do
4  |   if GPR is given then
5  |   |   split associated GPR by 'or' connectors to extract complex;
6  |   |   foreach complex do
7  |   |   |   split complex by the 'and' connector to extract associated genes;
8  |   |   |   foreach gene ∈ complex do
9  |   |   |   |   if gene ∈ ExpressionSet then
10 |   |   |   |   |   extract associated expression value from the ExpressionSet;
11 |   |   |   |   |   return expression value for selected gene
12 |   |   |   |   else
13 |   |   |   |   |   if organism and typeID is given then
14 |   |   |   |   |   |   identify associated pathways to gene;
15 |   |   |   |   |   |   select main pathway (more genes present in the model);
16 |   |   |   |   |   |   extract associated expression values to main pathway;
17 |   |   |   |   |   |   compute the selected metric for missing to all expression data associated to the selected
18 |   |   |   |   |   |   |   pathway
19 |   |   |   |   |   |   |   return 0
20 |   |   |   |   |   find the minimal expression value of all genes associated in the complex;
21 |   |   |   |   |   return identified min value
22 |   |   |   compute sum the minimal expression values associated to all genes included in the complex that conform the
23 |   |   |   |   GPR;
24 |   |   |   return computed sum
25 |   |   else
26 |   |   |   compute the selected metric for missing to all expression data included in the ExpressionSet;
27 |   |   |   return computed value
28 |   replace lower bound with computed value multiplied by -1;
29 |   replace upper bound with computed value
30 return the model with lower and upper bound modified

```

---

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