Supplementary Material

Supplementary Methods

1. PKU disease analysis by using Recon2.2 for a single compartment

First, we used the genome wide metabolic model (GeMM) Recon2.2[1] with simple medium composition in a single compartment setting (shown in Table S2). For the convenience of the calculation of amino acid metabolism, we only included essential amino acids and tyrosine in the medium; we did this by allowing minus the lower bound (i.e. import V_{max}) to be positive. We could neither find the exchange reaction ('EX_phpyr_e') nor the transport reaction ('PHPYRte') for phenylpyruvate in Recon2.2. As these two reactions are required for secreting phenylpyruvate into the urine, we added them to our version of Recon2.2. We then set biomass production, dopamine production, or biomass with dopamine production app://resources/notifications.html('biomass_reaction' + '3HLYTCL') as objective function, and did the respective FBA calculations.

In order to simulate PKU patients, we knocked out the gene 'HGNC:8582' (The 'PAH' gene which catalyzes both reaction 'r0399' and reaction 'PHETHPTOX2' in Recon2.2), and then ran the FBA again to check for changes in the value of the objective function. For simulating Tyr supplementation therapy, the lower bound of its exchange reaction was made more negative (i.e. the V_{max} of its uptake was increased) and the effect on the objective function computed. For simulating Phe deprivation, we increased the lower bound of its exchange reaction (i.e. reduced the V_{max} of its uptake).

2. Parameter settings for the 'Recon2.2plusthreeIndependent' model

For the three-compartment without-competition-for-transport model, we set the maximum flux ('reaction upper bound') of the biomass reaction in the liver to 0.05 and the maximum flux for the biomass reaction in the brain to 0.1. To simulate the normal case for tyrosine uptake from the diet, we provided 0.04 of tyrosine to the medium (by setting the lower bound for Tyr import to -0.04). With this, most Tyr still had to come from Phe. The production of phenylpyruvate should not be high as this will prevent Phe competition with Tyr to cross the BBB. We therefore set the upper bound of the transformation reaction of phenylalanine to phenyl ketone in the liver to 0.1 (i.e. the maximum phenylpyruvate production in the liver was 0.1). We set the liver to take up Phe at 0.2 (by changing the upper bound to -0.2; the lower bound was also -0.2), which was postulated to correspond to the daily Phe uptake from the food. We did not limit the Phe transport from blood to brain (i.e. the lower bound was left at -1000). All components of the 'medium' (Table S2) could only be taken up by the liver (the medium taken to represent the contents of the portal vein), with the exception of oxygen which could be taken up by the blood. All amino acids except for Phe, phenyl ketone, glucose, L-dopa, dioxygen, acetoacetate, R)-3-hydroxybutyrate, CO_2, protons, lactate, sulfate, urea, dopamine, adrenaline, serotonin, ammonium and hydrogen phosphate could be secreted from the blood (to the urine); other metabolites in the model could neither be secreted into nor taken up from the outside world.

With these settings, we carried out the FBA calculations with the sum of brain biomass, dopamine and serotonin production as objective function and used the value obtained for each of the three sub-objectives as 'essential need value' for our subsequent model calculations. We knocked out the gene 'HGNC:8582' to simulate metabolism in PKU patients. We then tried to supply extra tyrosine (by making the lower bound of its import

reaction more negative), with or without Phe-deprivation (which we simulated by making its import reaction bound less negative).

3. Parameter settings for the 'Recon2.2plusthreeCompetitive' model

For the three-compartment amino acid transport competition model 'Recon2.2plusthreeCompetitive', we again set the maximum flux ('reaction upper bound') of the biomass reaction in the liver to 0.05 (i.e. to simulate the biomass production in the liver) and highest flux for the biomass reaction in the brain to 0.1. We should set this value because when the system produced the same amount of brain biomass or dopamine or serotonin, the biomass required less transport ability. If we did not set this value, the model will try its best to produce just biomass, without requiring itself to make dopamine and serotonin. To simulate the normal case for tyrosine uptake from the diet and portal vein, we provided 0.04 or 0.1 of tyrosine through the medium to the liver (i.e. we set the lower bound (import V_{max}), for correspondence with the reduced tyrosine diet and the rich tyrosine diet, respectively. At this value, Tyr could also come from Phe. We set the upper bound of phenyl ketone secretion from the liver to 0.1, and the liver to take up Phe again at 0.2. Again, we did not allow Phe secretion through blood (i.e. the upper bound for reaction 'EX_phe_L_BD' was 0), otherwise there would be no competition. We also set the lowest need of serotonin to 0.095 (in order to limit the competition of Trp for the carrier). At the same time, we set the highest ability of the LAT transporter exit from the brain to blood side of the BBB to 0.198, which should cause the three amino acids and L-dopa to compete for entrance into the brain (see also below). All medium components (Table S2) could only be taken up by the liver, with the exception of O_2 which could be taken up by the blood. All amino acids except for Phe, phenyl ketone, glucose, L-dopa, dioxygen, acetoacetate, R)-3-hydroxybutyrate, CO_2, protons, lactate, sulfate, urea, dopamine, adrenaline, serotonin, ammonium and hydrogen phosphate could be secreted from the blood; other metabolites could not be exchanged with the environment.

With these settings, we did the FBA calculation with the sum of brain biomass, dopamine and serotonin production as objective function. Subsequently we used each of their values value as 'essential need value' for the model, thereby forcing FBA to synthesis all three commodities (i.e. brain biomass, serotonin and dopamine). We knocked out the gene 'HGNC:8582' (PAH gene) to simulate PKU patients. We then tried to supply extra tyrosine (by setting the Tyr uptake lower bound to -0.1 for tyrosine limited diet, and -0.2 for rich tyrosine diet), with or without Phe-deprivation.

4. Biomarker prediction for PKU disease with the models 'Recon2.2plusthreeCompetitive' and Recon2

4.1 With model 'Recon2.2plusthreeCompetitive' (amino acid transport competition)

We used the 'Recon2.2plusthreeCompetitive' model with PKU-specific parameters (e.g. competitive transport and multiple objectives in the objective function, see above) for biomarker discovery. To verify whether Phe is a biomarker or not, we assumed Phe could be secreted virtually from blood and did biomarker finding for the PAH gene. For other cases, Phe could not be secreted from blood, otherwise there should be no competition for Phe, Tyr and Trp for crossing the BBB.

In contrast to our group's previous study[2], in which virtual exchange reactions for potential biomarkers were *not* incorporated, this paper extends the methodology by allowing to add such a single virtual exchange reaction, i.e. one at a time for each potential biomarker. We selected 17 metabolites from the model and in 17 distinct analyses we incorporated an exchange reaction for one of them into the model, subsequently performing biomarker prediction for the PAH gene deletion. The metabolites were 5,6,7,8-tetrahydrobiopterin

(BH4), N-acetyl serotonin, melatonin, 6-Hydroxymelatonin, Formyl-N-acetyl-5-methoxykynurenamine, phenylacetyl-CoA (4-), 3-iodo-L-tyrosine, diiodine, (2-hydroxyphenyl) acetic acid, 3,4-dihydroxymandelaldehyde, phenylalanine product with tyrosine hydroxylase, 6,7-dihydrobiopterin (BH2), 4a-hydroxytetrahydrobiopterin, 2-phenylethanaminium, phenylacetaldehyde, phenylacetate and tirocinium, all in the brain. These metabolites are involved in neurotransmitter and phenylpyruvate metabolism. The results (Table S5A) show that the incorporation of these virtual reactions allows for a much wider biomarker prediction. It also allows for predictions in changes of the levels of various neurotransmitters and other functional molecules and therefore of aspects of brain function. For biomarker finding, we knocked out the PAH gene (i.e. the reactions from Phe to Tyr were knocked out.) and set 100% objective function to attain. We used the same medium composition as we did in the predictions for PKU disease with the 'Recon2.2plusthreeCompetitive' model (Table S2). For identification of biomarkers, the FVA results of flux through the metabolite's exchange reaction before and after knocking out the PAH gene, were compared. Only metabolites with a score (Shlomi et al[3]) in excess of 0.1 were selected.

4-2 With model Recon2 only

We also checked whether we could find the biomarkers for the PAH gene with the Recon2 model which Thiele et al used in 2013. For exchange reactions in Recon2, we set each lower bound to '-1' and left the upper bound at 1000 (unlimited). We chose biomass production as objective function and forced flux through the pathway from Phe to Tyr in healthy case. There were two reactions ('PHETHPTOX2' and 'r0399') to catalyze Phe to Tyr in Recon2, with different genes. We knocked out 'PHETHPTOX2' and did biomarker finding for reaction 'r0399' with 100%, 90%, 50% and 0 of objective function to attain.

Supplementary Results

1. Single-compartment model of PKU and its potential therapies

In our attempt to describe PKU disease, we began by using Recon2.2 in a single compartment setting, with biomass as the objective function (Figure S1). The thick colored lines in this figure show the main fluxes we thereby predicted through the reactions indicated by the letters alongside; the magnitudes of the fluxes are given by the numbers to the right of the lettering. Reactions predicted not to carry flux are indicated by very thin gray lines. Even though the calculations were performed for the entire Recon2.2 network, we only show the results for a few, most relevant, reactions. The metabolites are indicated by the orange circles with their abbreviated names alongside. Figure S1 shows that for this model of individuals not affected by PKU disease, the FBA calculated substantial flux (0.169) to biomass, with some flux (0.169) diverting to fumarate, but no flux to phenylpyruvate, dopamine or serotonin. We then deleted the PAH gene to simulate PKU patients. Figure S1B shows the effect of deleting both reactions catalyzed by its gene products (see the two red crosses in Figure S1B): a higher phenylpyruvate production (0.156 (Figure S1B) rather than 0.00 (Figure S1A)), which is in line with the classical PKU phenotype. However, the biomass synthesis (0.169) was the same as in case of fully active conversion of Phe to Tyr (compare Fig. S1B to Fig. S1A), which is not realistic. Moreover, although we found that Phe deprivation decreased the phenylpyruvate production (down from 0.156 to 0.006), it did not enhance biomass production (Figure S1D). Tyr supplementation did not have any such

potentially curative effect (Figure S1C). When we reduced the Tyr supply to both the healthy-person model and the disease model, we found that PAH gene deletion would reduce biomass synthesis (Figure S2B), but then Tyr supplementation alone to the patient was predicted to restore biomass production, whilst Phe deprivation alone would not (See Figure S2C and Figure S2D).

The predictions of this single-compartment model are thereby not in line with observations in PKU disease or its therapies: unusually small head size (microcephaly)[4] and retarded mental development occur in untreated PKU patients, which would have been predicted by a diminished biomass synthesis. Moreover, the prediction that in case of limited daily Tyr uptake Tyr supplementation should cure the disease is incorrect.[5-6] And, it is Phe restriction rather than Tyr supplementation that manages PKU disease successfully.[7-8]

Tyr is the precursor of the neurotransmitters dopamine and adrenaline (epinephrine), which are important for the reward and activity systems of brain function[9-10] and may thereby be necessary for the cognitive development of the brain. And indeed, it is this cognitive development that seems to be impaired in the disease. In order to examine if the one-compartment-Recon2.2 model can reproduce this, we changed the objective function to dopamine production. As shown in Figure S3, we found that although there was dopamine production in the healthy case (Figure S3A) and lack thereof in the PAH deletion case (Figure S3B) supplementation of tyrosine to the model again solved this problem (Figure S3C) whilst Phe deprivation did not improve dopamine production (Figure S3D). Now there was no biomass production, neither in the healthy condition nor in the PKU condition (Figure S3A-D). As PKU pathology develops at an age where the brain is still growing in volume,[11] we did not consider this to be quite realistic. We then used the sum of biomass and dopamine synthesis as the 'combined objective function' for the FBA, as discussed under 'Methods'. If thus requested to perform maximally, the network would produce 0.169 units of biomass and 0.169 units of dopamine which we then assumed to represent the essential need of a simulated healthy person. Figure S4A also shows that for this to be affected, the model simulating the healthy person should take up 0.2 Phe and 0.015 Trp from the medium and re-reduce the coenzyme dihydrobiopterin at a rate of 0.325. In this simulation, some 78% of the Phe taken up was converted to Tyr by phenylalanine hydroxylase and the rest to biomass; there was no flux to phenylpyruvate ('phpyr'). Part of the Tyr was then converted to L-dopa at a flux rate of 0.169. This flux could continue to dopamine, nor-epinephrine and epinephrine (=adrenaline), which was also allowed to exit the brain cells. We take these results as a fair simulation of the essentials of a healthy person.

Figure S4B suggests that people deficient in the PAH gene will again be in trouble: less dopamine production, consistent with reduced development of cognitive functions and a somewhat reduced growth rate. Phenylpyruvate is now secreted by the model, in line with the initial diagnostic of the disease.[12-13] Since PAH makes Tyr and Tyr should thereby be reduced in PAH deprived individuals, one might expect Tyr supplementation of the nutrition to cure the disease. Indeed, when we only provided extra tyrosine to these *in silico* PKU patients, we found the biomass and dopamine production to be recovered (Figure S4C). This shows that with the combined objective function, FBA of the single compartment model does predict a brain-specific pathology for PAH dysfunction (reduced intellectual development due to reduced dopamine and epinephrine levels) as well as a therapy, i.e. Tyr supplementation: an apparent success for the methodology.

However, the prediction of a successful Tyr-supplementation-only therapy, as consistent with intuition as it may be, is known to be a fallacy: Tyr supplementation through nutrition alone does *not* cure the disease.[8,14] This made us re-think the rationale of our single compartment FBA approach.

2. Three compartments model without competition of transport and its potential therapies

As dopamine production occurs in the brain,[15-16] we modified our single compartment Recon2.2 model, by making the brain an explicit compartment. This turned the metabolic model into one consisting of three compartments, i.e. the liver where all of Recon2.2 was considered to be active, the blood without biochemical activities but into which the liver could secrete its products and from which the brain could take up some, and the brain also with a whole Recon2.2 network. FBA of this three compartments model with a combined biomass objective of growth of the brain, serotonin production, as well as consumption of epinephrine and dopamine by the brain, then produced inhibition of that objective function by deletion of PAH from the liver. However, Tyr supplementation again restored dopamine and biomass production in the brain as well as this objective function (see Figure S5C), falsely predicting that supplementation with Tyr alone should cure the disease. This three-compartment model could not predict the therapeutic effectiveness of Phe deprivation either (see Figure S5D).

3. Three compartments model with competition for Tyr hydroxylase

In the literature it has been amply suggested that increased Phe and phenylpyruvate concentrations are toxic to the brain by inhibiting Tyr hydroxylase and that this is responsible for the pathology of PKU.[17-19] The existing FBA methodology cannot deal with kinetic phenomena such as competitive inhibition and we therefore had to devise a modification of the FBA procedure. Competition by Phe should reduce the free enzyme concentration of Tyr hydroxylase that is available to react with Tyr. In order to simulate this, we made the catalytic cycle of Tyr hydroxylase explicit, with conversion of Tyr to levodopa bringing the enzyme in a different state (i.e. from TH_B_INB to TH_A_INB) plus a reaction in which the enzyme returned to its original state. We then added a 'virtual' reaction where brain Phe would be converted to metabolite 'PheP_INB', whilst also brings the enzyme in its used state. Phe could also dissociate from 'PheP_INB' through reaction 'PhePdePhe_INB'. When the flux for Phe from blood to brain exceeds 0.8, the flux must go through reaction 'Phe_TH_INB'. By limiting the maximum rate at which the enzyme could return to its original state, we reduced the amount of enzyme available for Tyr, thereby simulating the competition between Phe and Tyr for this enzyme.

Still using the three compartments model with independent pathways for Phe, Tyr and Trp to move into the brain, we then calculated that PAH inactivation increased the flux through which Phe transiently combined with tyrosine hydroxylase (Compare Figure S6B with Figure S6A) and reduced the flux at which Tyr did this with as consequence that the flux to dopamine decreased, without effect on biomass production. Phe deprivation was calculated to reduce the flux through Phe combining with tyrosine hydroxylase (Figure S6D), but it did not enhance dopamine production in the brain. Even though the dopamine production did not increase much by providing the Tyr, in this model Tyr supplementation of the food could still alleviate mental development by taking more tyrosine into the brain (Figure S6C): also this model with competition for Tyr hydroxylase failed to represent reality. Consequently, these computations alerted us to the fact that this toxicity mechanism[17-19] alone may neither explain PKU disease nor its therapies.

4. Three compartments model with competition for amino acid transport, based on Recon3D instead of Recon2.2

In the main text we show that the above Recon2.2-based three compartments model with competition of amino acids for transport across the BBB (all as formulated in the 'Recon2.2plusthreeCompetitive model') does simulate the pathology and therapies of PKU. It also produced correct biomarker predictions, which constitutes an advance over the absence of such biomarker prediction for PKU in 2013[20]. We used Recon2.2 to highlight the cause of the improvement of PKU biomarker prediction. However, Recon2.2. has been generally superseded by Recon3D[19] and we therefore checked that a 3-compartment Recon 3D model with amino acid competition for transport across the BBB produced the same results as Recon2.2.

4.1 Amino acids competition for transport in Recon3D

To assess the robustness of our approach towards changes in the metabolic map, we created a three-compartment model by using the more recent Recon3D[21] and followed the same methods as we did above for the Recon2.2plusthreeCompetitive. We then used this 'Recon3DplusthreeCompetitive' model to demonstrate the symptoms and treatment options for PKU disease. As shown in Figure S12, in a healthy individual, the objective of brain biomass plus dopamine plus serotonin production had a value of 0.252 (biomass_reaction_INB (0.1) + 3HLYTCL_INB (0.057) + 5HLTDL_INB (0.095)). In simulated PKU patients, we observed a decrease in dopamine and brain biomass, and an increase in the secretion of phenylpyruvate or its derivative (Figure S12B). Again, there was no improvement when we only provided tyrosine in the medium (Figure S13). Phe deprivation did decrease the production of phenylpyruvate and increase the production of brain biomass, dopamine and serotonin (Figure 12D). We conclude that the results obtained with the Recon3D version of the model were qualitatively identical to those shown in the main text for the Recon2.2 version.

4.2 Phe-restricted diet and obesity in the 'Recon3DplusthreeCompetitive' model

We compared the relationship between a Phe-restricted diet and obesity, now using the 'Recon3DplusthreeCompetitive' model, with TAG production again serving as a representation of obesity. As shown in Figure S14, an excess of glucose (carbohydrate) intake is again predicted to cause obesity in both healthy individuals and PKU patients. As PKU patients tend to shift their energy consumption from protein to carbohydrate, this may help warn against obesity in PKU patients on Phe deprivation diet. Also, according to the 'Recon3DplusthreeCompetitive' model, such PKU patients should be able to control their weight by limiting carbohydrate intake (Figure S13D).

4.3 Alternative therapies for PKU patients

We then utilized the 'Recon3DplusthreeCompetitive' model to identify potential therapies for PKU patients. Again, since our model only included three compartments, we used liver biomass synthesis to represent the biomass synthesis outside of the brain. As shown in the Figure S15, the objective function improved when we increased the liver biomass synthesis, suggesting that exercise may be beneficial to PKU patients. Additionally, we also increased the BBB amino acid transport ability for PKU patients and observed an increase in brain biomass production, dopamine production and serotonin production (Figure S16). However, phenylpyruvate flux remained high and phenylpyruvate and Phe may compete with tyrosine for tyrosine hydroxylase, affecting dopamine production. It is important to carefully consider the impact of high phenylpyruvate levels on PKU patients.

5. A tissue specific model: 'Brain_liver_specific_plusthreeCompetitive' model

5.1 Amino acids competition for transport

In addition to utilizing the genome (DNA)-based global human metabolic models Recon2.2 and Recon3D, we also constructed a three-compartments model by starting from an existing, expression based, tissue specific model. The brain and liver tissue specific models were sourced from the literature[22-23] and incorporated through the same methodology. We then used the new 'Brain_liver_specific_plusthreeCompetitive' model to simulate the symptoms of and treatment options for PKU disease. As depicted in Figure S17, we obtained for the objective of brain biomass plus dopamine plus serotonin production a value of 0.252 (biomass_reaction_INB (0.1) + 3HLYTCL_INB (0.057) + 5HLTDL_INB (0.095)) in healthy individuals. In PKU patients, we observed a decrease in dopamine and brain biomass, along with an increase in phenylpyruvate secretion (Figure S17B). Tyr supplementation alone was not sufficient to cure the disease (Figure S18). Phe deprivation decreased phenylpyruvate production whilst increasing brain biomass, dopamine and serotonin production. Because we calculated this for the case that the patients did not take enough Tyr from their diet, extra Tyr was here required for optimal Phe-deprivation treatment (Figure S17D).

5.2 Phe-restricted diet and obesity

We also utilized the tissue-specific model to understand the underlying mechanisms by which a Phe-restricted diet can lead to obesity. We used the TAG production as objective function to represent obesity after the model had satisfied essential needs. As demonstrated in Figure S19, excessive glucose (carbohydrate) uptake will lead to obesity for both healthy individuals and PKU patients. However, it is relatively easier for healthy individuals to control their glucose uptake as compared to PKU patients and therefore this implies an increased risk of obesity for the latter. Of course, PKU patients could also control their weight if they limited their carbohydrate intake (Figure S19D).

5.3 Alternative therapies for PKU patients

We employed the 'Brain_liver_specific_plusthreeCompetitive' model to identify the potential therapies for PKU patients. As our model only incorporated three compartments, we used liver biomass synthesis as a proxy for synthesis in other part of the body. As illustrated in Figure S20, we observed an improvement in brain biomass, dopamine production and serotonin production when we increased liver biomass synthesis. This again suggests that exercise may benefit PKU patients.

We also raised the BBB amino acid transport ability in a new simulation of PKU patients. The reduction in dopamine and biomass production caused by PAH inactivation was reversed (Figure S21), but the phenylpyruvate flux remained high and could potentially compete with tyrosine hydroxylase and affect dopamine production. Thus, before implementing this treatment clinically, the potential effects of a high phenylpyruvate levels on patients should be considered.

6. Choice of objective in FBA

In the above and the main text, we assumed that all sub-objectives in the objective function (i.e., biomass, dopamine and serotonin) carry equal weight. However, we also experimented with various combinations of contributions for the objectives at two different concentrations of Tyr and here report the results for the various models.

6.1 Different contributions of the various objectives, compared for the non-competitive model

Except for the unrealistic combination (1·biomass+3·dopamine+2·serotonin), we found that Tyr supplementation alone cured the disease and Phe deprivation alone did not improve the symptoms of PKU (Table S3 excel in addition Supplementary material) no matter Tyr was sufficient or insufficient. We have also tried to establish a minimum value for serotonin production, which is to check whether the non-competitive model could address PKU disease and its therapies. We could not obtain the correct result by any change. These results again suggest that the non-competitive model cannot be used to address PKU disease.

6.2 Different contributions of the various objectives, compared for the transport-competition model

In the main text, we assumed that all objectives in the objective function carried the same weight. However, we also calculated with various sub-objective weights at three different concentrations of Tyr. When Tyr was sufficient for biomass synthesis (total maximum 0.15, i.e. Tyr=0.04 and 0.1) and Tyr had the same or a smaller weight than serotonin in the objective function, we observed consistent results regardless of the objective combination we chose: both Phe deprivation and Phe deprivation with Tyr supplementation were effective in curing PKU, while Tyr supplementation alone did not provide any benefits (Table S4 excel in in addition Supplementary material). When Tyr had a higher contribution than serotonin in the objective function, we found Tyr supplementation could increase the PKU symptoms which however reduced the serotonin production. To redress this issue, we set a minimum value for serotonin production. However, when tyrosine was insufficient for biomass synthesis, we found that increasing the Tyr concentration in the system resulted in a decrease in serotonin production and an increase in brain biomass production, regardless of the objective combination. This was because the same amount of brain biomass synthesis utilizes less transporter as compared with the same amount of dopamine production or serotonin production. Furthermore, we found that Phe deprivation alone did not improve the symptoms of PKU. To address this issue, we established a minimum value for serotonin production. After that, we found essentially the same results as in the main text for the three-compartments amino-acid-transport-competition model.

7. Parameter choice (taking the values of transporter parameters as an example)

The choice of parameter values can greatly impact the model's predictions. In this paper, we employed a competitive transport mechanism to investigate the PKU disease. We chose the transporter ability to perform an analysis of the sensitivity of our prediction to its parameter value. To do this, we first raised the transport ability (in the sense of the upper bound of the outward movement of the empty transporter across the BBB) from 0.198 to 0.25. As depicted in Table S7 (Supplementary material), we found that supplementing with Tyr alone was predicted to cure PKU disease, whilst Phe deprivation was predicted not to provide any benefit for PKU patient. These results were not consistent with actual clinical observations. This we took as confirmation that competition for amino acid transport, which should be alleviated by increasing the upper bound of the outward movement of the empty transporter across the BBB), is essential for the model explaining the experimental observations. Indeed, when we decreased the transport ability from 0.198 to 0.1 (Table S7), we observed that the competition mechanism still existed and we could also demonstrate that Tyr supplementation alone is ineffective for PKU patients, whilst Phe deprivation was necessary to improve the PKU symptoms. However, there was no dopamine observed in any of the cases, which is not biologically realistic.

8. Affinity coefficients and FBA

Affinity coefficients cannot be put into FBA calculations directly, so one would need a work-around. In a linear pathway a reduced affinity of an enzyme for its substrates should have no effect anyway on the flux. Affinity changes (through point mutation in the active site of the enzyme) should have an effect however at branch points in the pathway, favoring flux into one branch over flux into the other. Our workaround would be to modify the reaction bounds accordingly. Combining with dynamic FBA, we could further estimate concentration changes through flux calculations over time.

Supplementary Figures

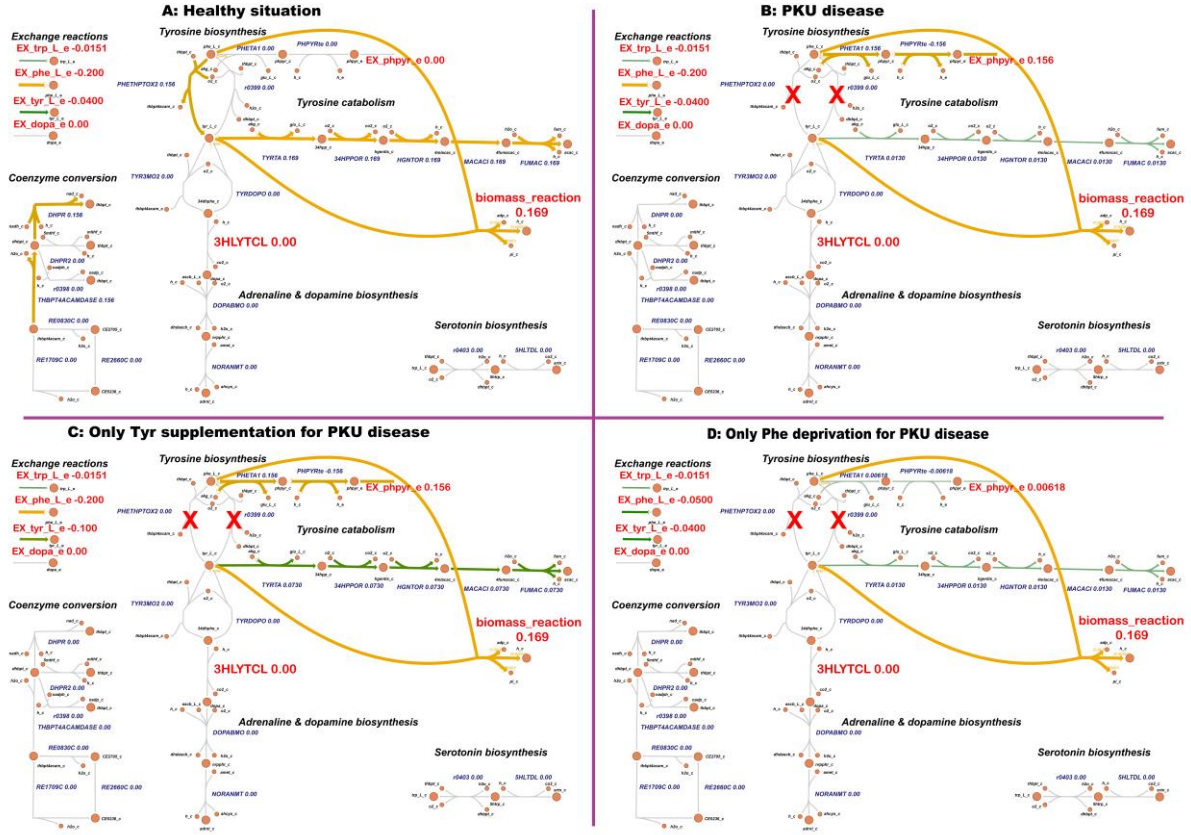

Figure S1. FBA results for four instantiations of the single compartment Recon2.2 model of PKU disease and its therapies. Medium Tyr was taken high enough for biomass synthesis; cf Figure S2. Biomass production was taken as objective function. (A) represents the healthy situation. (B-D) represent PKU disease resulting from knocking out the PAH gene (i.e., both reactions that the gene product catalyzes: PHETHPTOX2 and r0399). (C) is an attempt to simulate an intuitive (yet ineffective) therapy for PKU disease consisting of adding extra tyrosine to the medium (i.e. making the lower bound for Tyr uptake more negative) and (D) is an attempt to simulate the actually effective therapy for PKU disease consisting of a mere decrease in the absolute magnitude of the bound for Phe uptake from the medium. For each reaction, the calculated flux is written next to the name of the reaction and this is enlarged for the more important reactions. Exchange fluxes are negative when they correspond to uptake and positive for secretion. A red cross means that the corresponding reaction has been

blocked. The green and orange colors of lines mean that the corresponding reaction carries flux, more flux through the orange than through the green reactions. The 3HLYTCL flux is taken to represent the dopamine synthesis flux.

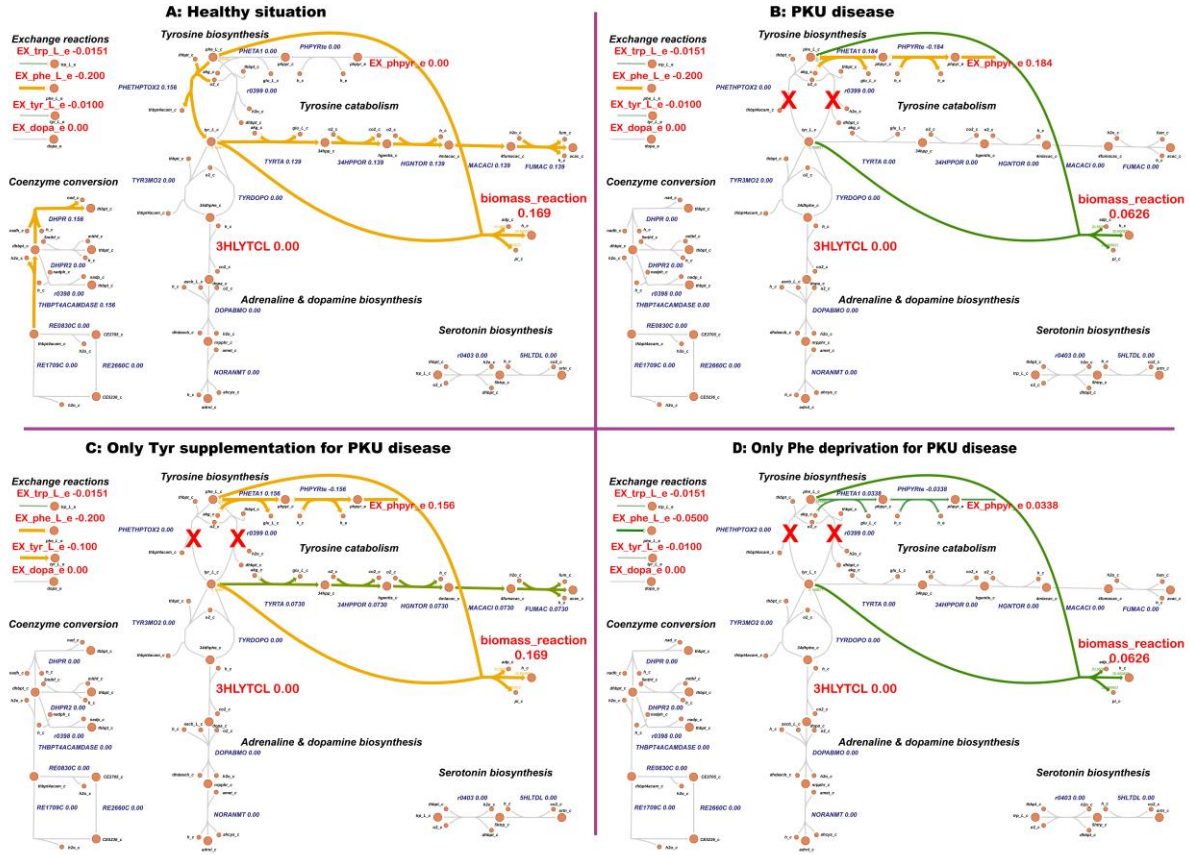

Figure S2. FBA results for four instantiations of the Tyrosine-limited version of the single compartment Recon2.2 model of PKU disease and its therapies (Here medium Tyr was taken smaller than in Figure S1, i.e. not enough for biomass synthesis). Biomass production was taken as objective function. (A) represents the healthy situation, (B-D) represent PKU disease resulting from knocking out the PAH gene, (C) simulates the intuitive but ineffective therapy for PKU disease consisting of adding extra tyrosine to the medium and (D) simulates the effective therapy for PKU disease by only decreasing Phe in the medium.

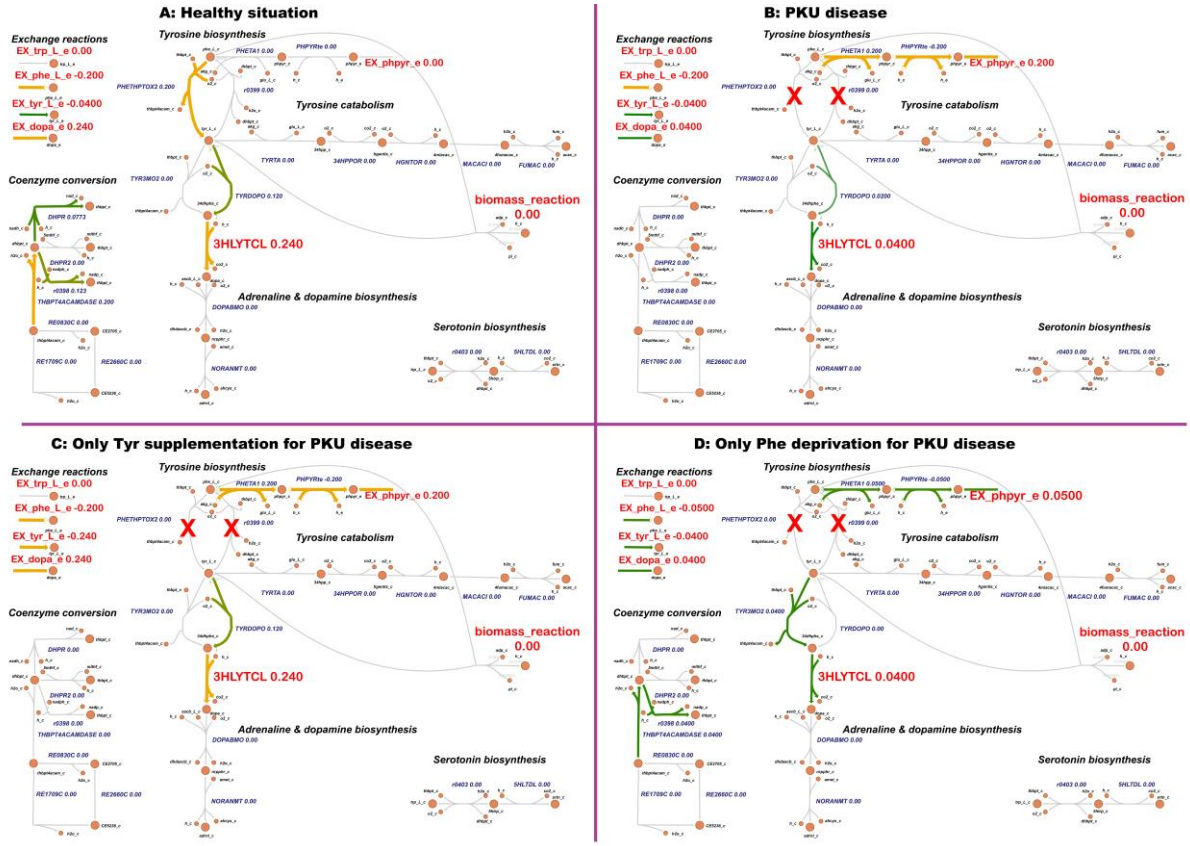

Figure S3. FBA results for four instantiations of the single compartment Recon2.2 model of PKU disease, now with dopamine production as objective function. (A) represents the healthy situation, (B-D) represent PKU disease by knocking out the PAH gene. (C) simulates a supposed (but actually ineffective) therapy for PKU disease consisting of adding tyrosine to the nutrition. (D) simulates the effective therapy for PKU disease by only decreasing Phe in the medium. When the dopamine was produced it was secreted out of the cells or transformed to adrenaline.

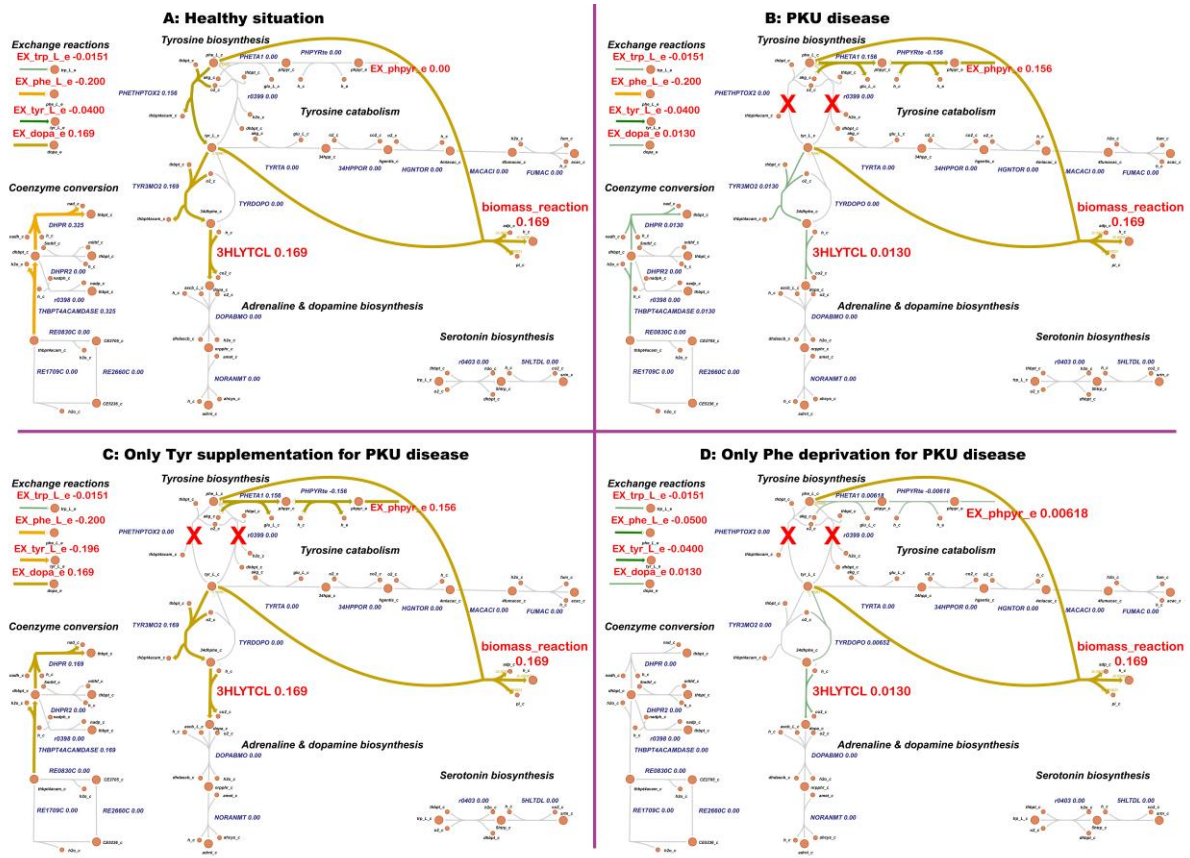

Figure S4. FBA results for four instantiations of the single compartment Recon2.2 model of PKU disease, with objective function = biomass reaction + dopamine production. (A) represents the healthy situation, (B-D) represent PKU disease by knocking out the PAH gene, (C) simulates a supposed therapy for PKU disease consisting of elevated tyrosine in the nutrition and (D) simulates the effective therapy for PKU disease by only decreasing Phe in the medium.

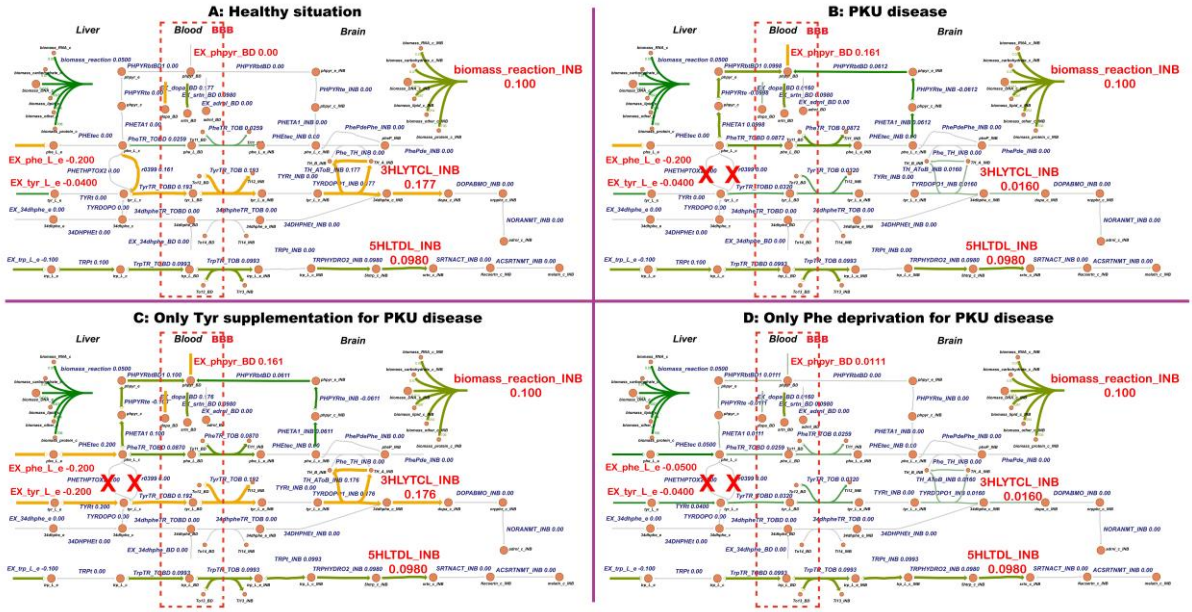

Figure S5. Four instantiations of the 'Recon2.2plusthreeIndependent' model with independent transport pathways for amino acids crossing the BBB, and without considering

competitive inhibition of tyrosine hydroxylase by Phe. The objective function = biomass production + dopamine production + serotonin production, all in brain. The left-hand line of the dashed line box represents the membranes between liver and blood, whilst the right line of the dashed line box represents the blood brain barrier (BBB). (A) represents the healthy situation, (B-D) represent the simulation of PKU disease by knocking out the PAH gene, (C) is the supposed treatment for PKU disease consisting of providing extra tyrosine and (D) is the supposed treatment of PKU disease by only decreasing the Phe in the nutrition. '_INB' and '_BD' refer to reactions and metabolites in brain and blood, respectively. The 3HLYTCL_INB flux is taken to represent the brain dopamine synthesis flux and 5HLTDL_INB the brain serotonin synthesis flux.

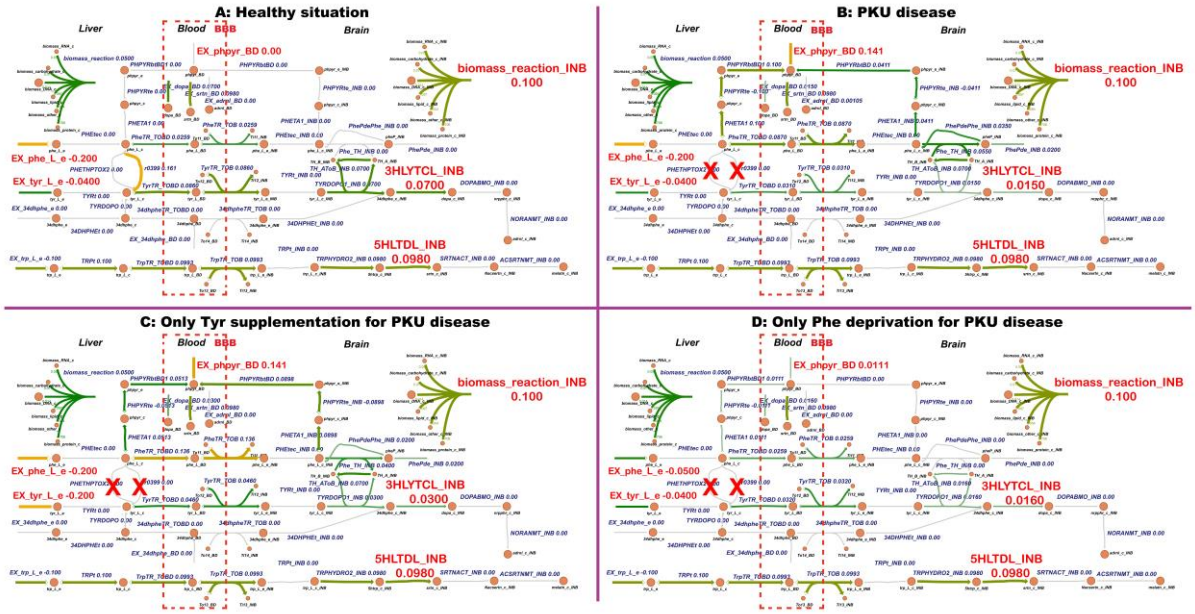

Figure S6. Four instantiations of 'Recon2.2plusthreeIndependent' with independent transport pathways for amino acids crossing the BBB, whilst considering competitive inhibition of tyrosine for tyrosine hydroxylase by Phe. The objective function = biomass production + dopamine production + serotonin production, all in brain. (A) represents the healthy situation, (B-D) represent the simulation of PKU disease by knocking out the PAH gene, (C) simulates the ineffective therapy for PKU disease consisting of providing extra tyrosine and (D) simulates the effective therapy for PKU disease by only decreasing the Phe in the medium.

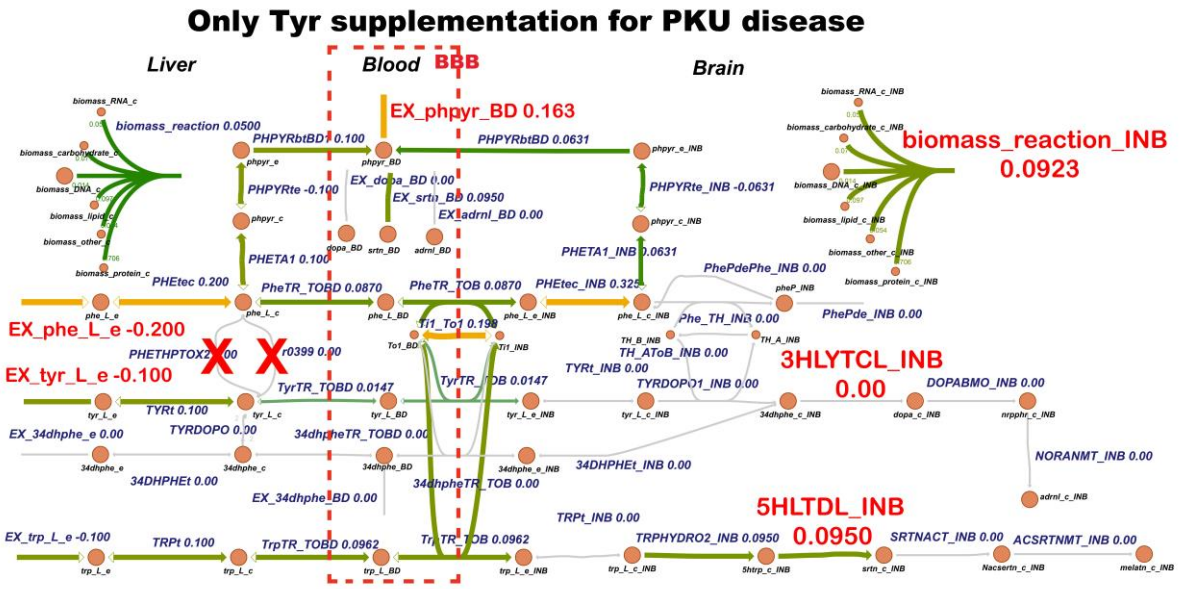

Figure S7. Diagram of the model with competition of the aromatic amino acids for transport across the BBB. Model 'Recon2.2plusthreeCompetitive' is an attempt to simulate the therapy for PKU disease consisting of only adding extra Tyr (i.e. increasing the Tyr import flux) in the medium (Objective function = biomass production + dopamine production + serotonin production, all in the brain).

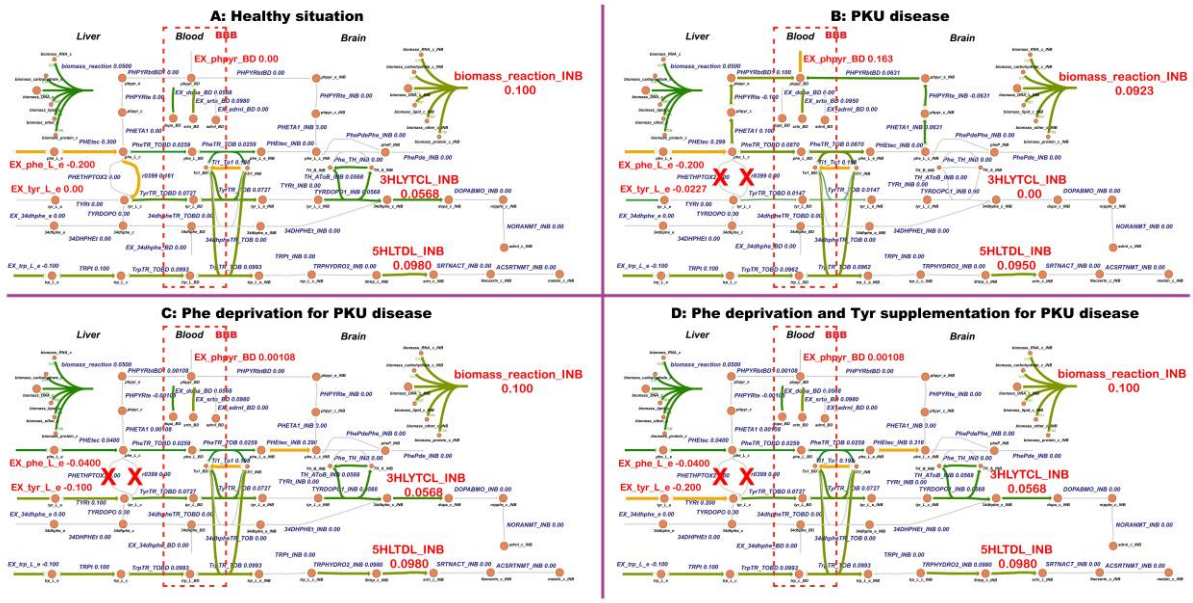

Figure S8. Four simulations by using the model 'Recon2.2plusthreeCompetitive' in diet with enough Tyr (i.e. 0.1 in the medium) (Objective function = biomass production + dopamine production + serotonin production, all in brain). (A) represents the healthy situation, (B-D) represent the simulation of PKU disease by knocking out the PAH gene, (C) simulates the effective therapy for PKU disease by only decreasing Phe in the medium and (D) simulates the ineffective therapy with extra Tyr for PKU disease consisting of providing extra Tyr and decreasing Phe in the medium.

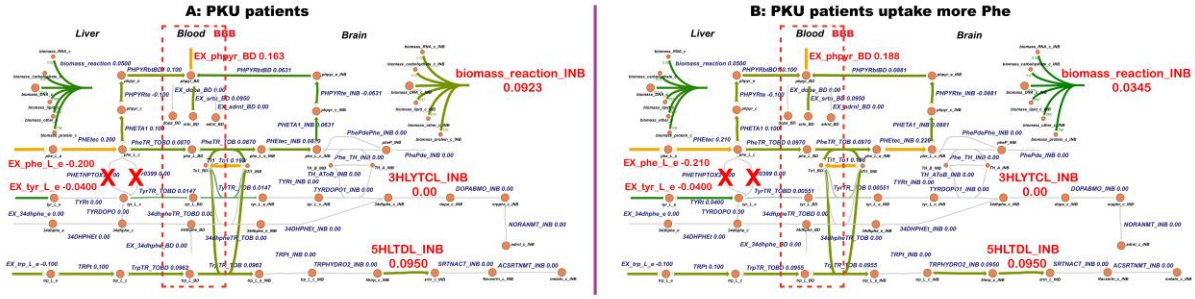

Figure S9. PKU patients taking more rather than less Phe as simulated using model 'Recon2.2plusthreeCompetitive' (Objective function = biomass production + dopamine production + serotonin production, all in brain). (A & B) represent the simulation of PKU disease by knocking out the PAH gene and (B) simulates the PKU patients taking more Phe.

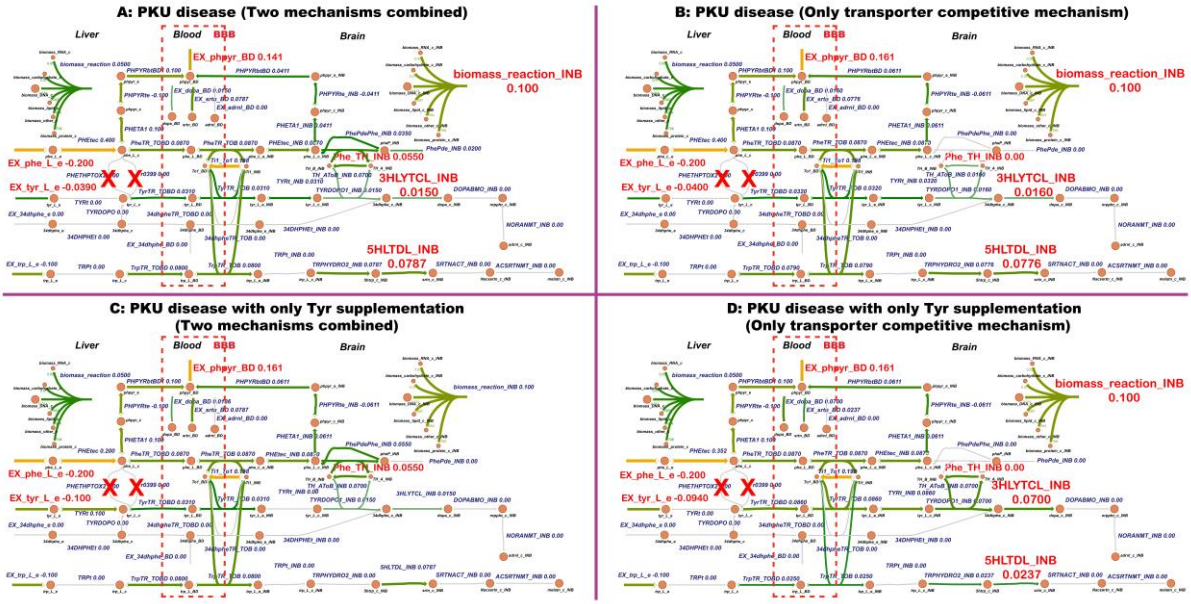

Figure S10. Combining the transporter competition mechanism with the Tyr hydroxylase competitive mechanism using the model 'Recon2.2plusthreeCompetitive' (Objective function = biomass production + dopamine production + serotonin production, all in brain). (A) represents the simulation of PKU disease by knocking out the PAH gene with both the transporter competition mechanism and the Tyr hydroxylase competition mechanism in place, (B) represent the simulation of PKU disease by knocking out the PAH gene with only transporter competitive mechanism, (C) simulates the ineffective therapy for PKU disease by only providing extra Tyr in the medium with both the transporter competition mechanism and the Tyr hydroxylase competition mechanism in place and (D) simulates the ineffective therapy for PKU disease of Tyr supplementation with only the transporter competition mechanism in place.

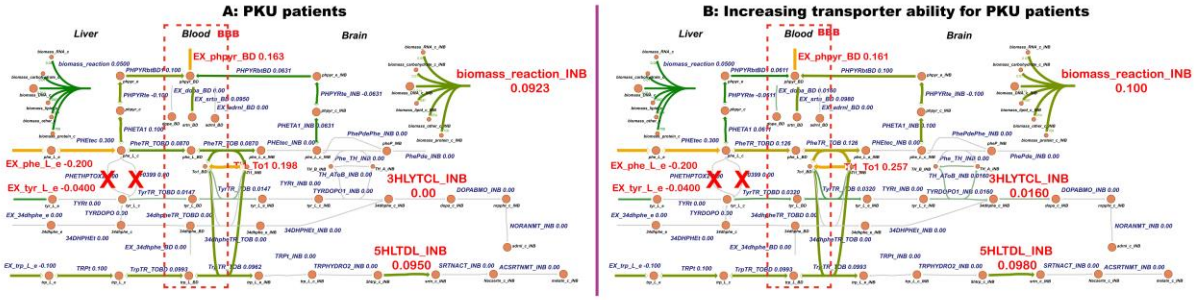

Figure S11. The FBA result of increasing the transporter ability through the BBB for PKU patients (i.e. increasing the upper bound for reaction 'Ti1_To1' from 0.198 in A to 0.257 in B) whilst using the 'Recon2.2plusthreeCompetitive' model (Objective function = biomass production + dopamine production + serotonin production, all in brain).

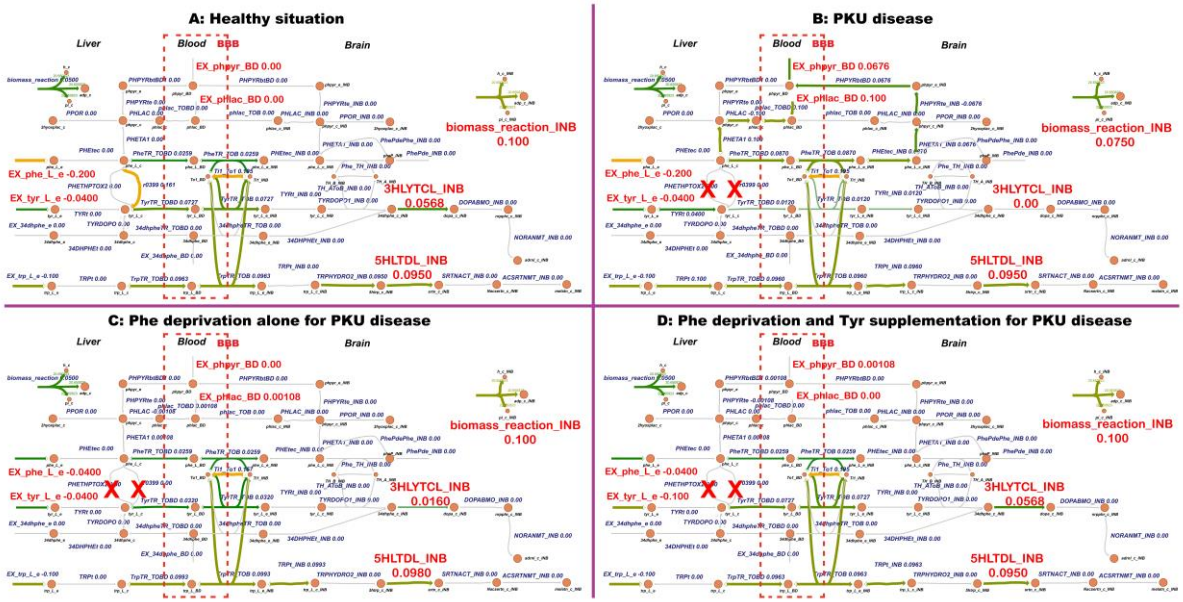

Figure S12. Four instantiations of the model 'Recon3DplusthreeCompetitive', with Objective function = biomass production + dopamine production + serotonin production, all in brain). (A) represents the healthy situation, (B-D) represent the simulation of PKU disease by knocking out the PAH gene, (C) simulates the effective therapy for PKU disease by only decreasing the Phe in the medium and (D) simulates the therapy for PKU disease consisting of providing extra Tyr whilst decreasing Phe in the medium.

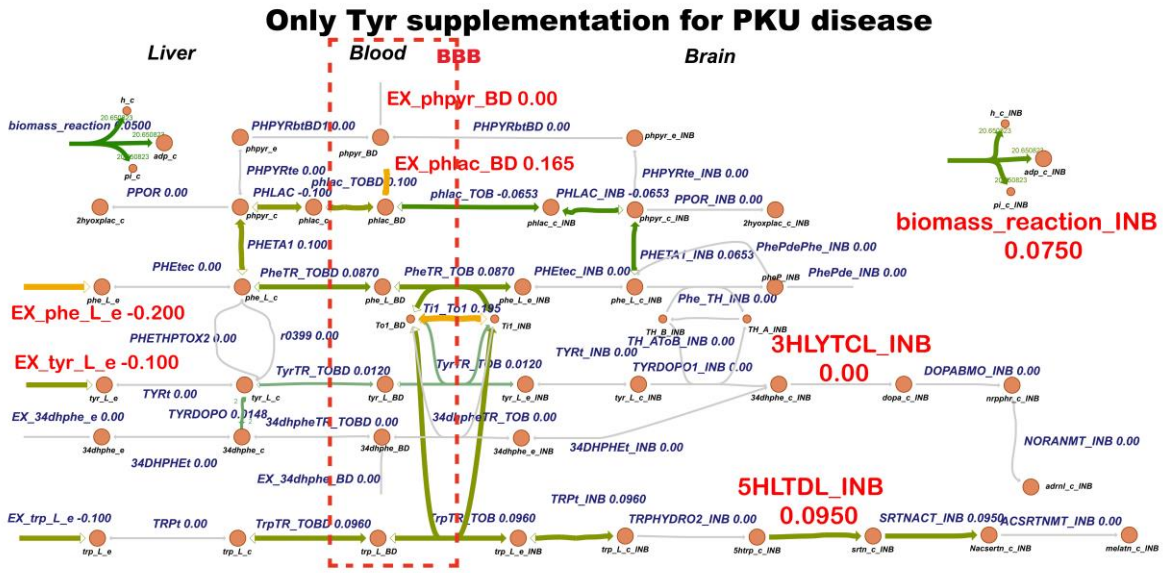

Figure S13. Model 'Recon3DplusthreeCompetitive' is an attempt to simulate the therapy for PKU disease consisting of only adding extra Tyr (i.e. increasing the Tyr import flux) in the medium (Objective function = biomass production + dopamine production + serotonin production, all in the brain).

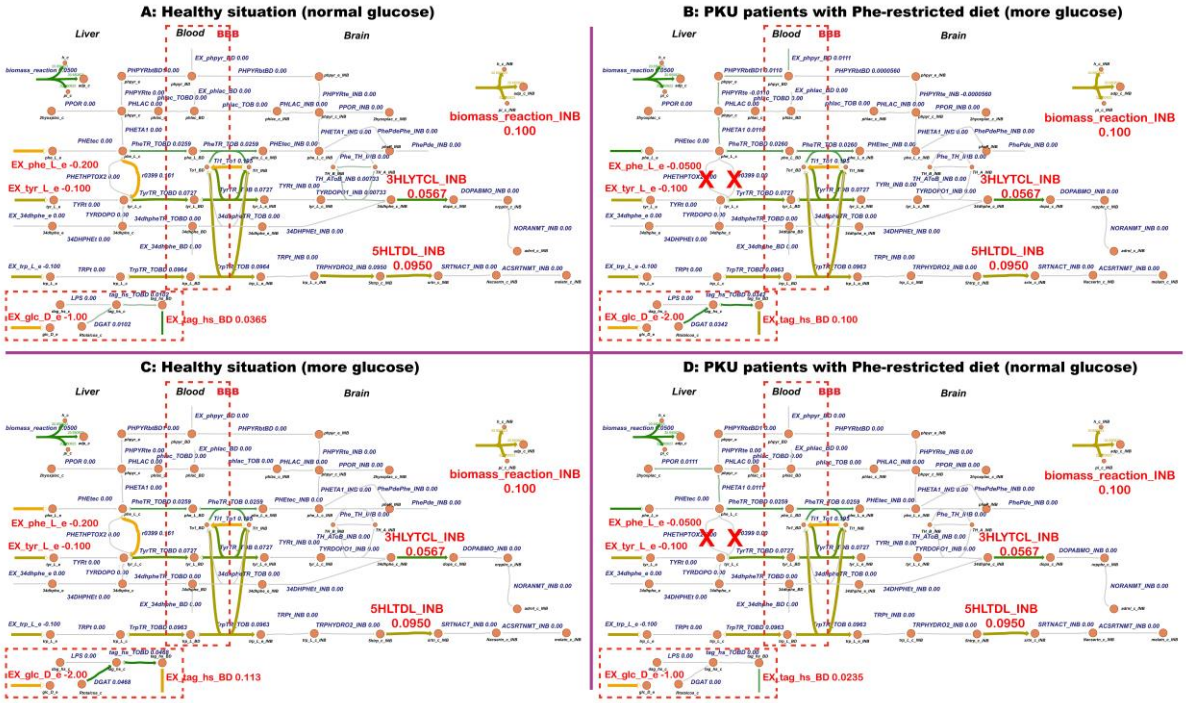

Figure S14. The triglyceride production in liver (see (EX_Tag_hs_e at the bottom left) predicted for (A) healthy subjects with normal glucose uptake, (B) PKU patients with Phe-restricted diet (including high glucose uptake), (C) healthy persons with high glucose uptake and (D) PKU patients with Phe-restricted diet at normal glucose uptake. The objective function was triglyceride production whilst the model was forced to satisfy what we defined as 'the essential needs' (a biomass reaction in brain of 0.1, a dopamine production of 0.057 and a serotonin production of 0.095). The 'Recon3DplusthreeCompetitive' model was used for these computations.

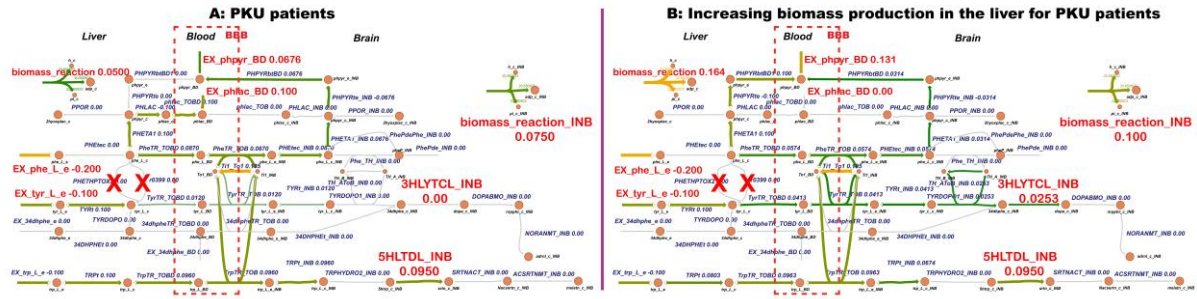

Figure S15. B versus A: The FBA result of increasing the biomass synthesis in the liver of PKU patients (i.e. simulated by increasing the upper bound for the 'biomass_reaction' in the liver) (Objective function: biomass reaction in brain (biomass_reaction_INB) + dopamine production (3HLYTCL_INB) + serotonin production (5HLTDL_INB)). The 'Recon3DplusthreeCompetitive' model above was used for these computations.

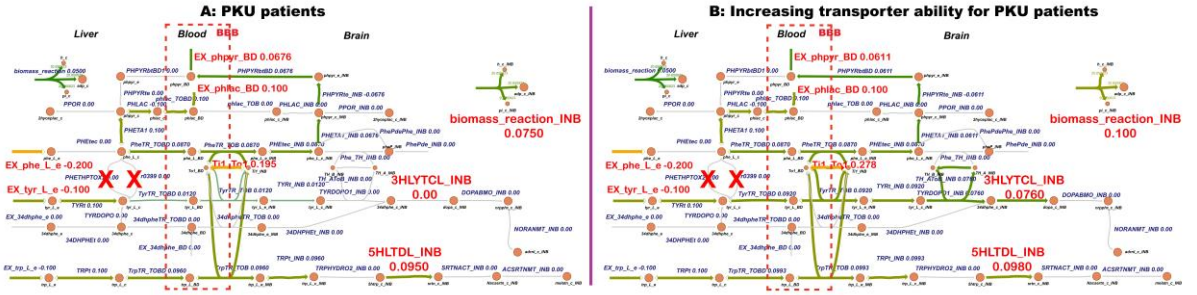

Figure S16. B versus A: The FBA result of increasing the transporter ability through BBB for PKU patients (i.e. increasing the upper bound for reaction 'Ti1_To1' from 0.195 to 0.278) (Objective function: biomass reaction in brain (biomass_reaction_INB) + dopamine production (3HLYTCL_INB) + serotonin production (5HLTDL_INB). The 'Recon3DplusthreeCompetitive' model was used for these computations.

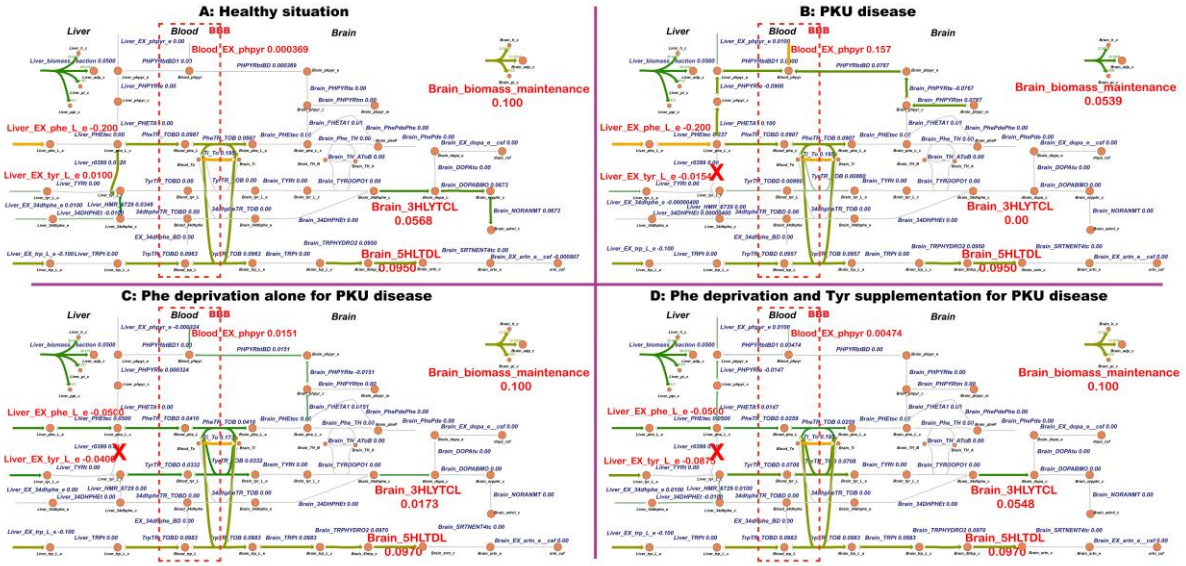

Figure S17. Four instantiations of the gene-expression-based tissue-specific model 'Brain_liver_ specific_plusthreeCompetitive' (Objective function = biomass production + dopamine production + serotonin production, all in brain). (A) represents the healthy situation, (B-D) represent the simulation of PKU disease by knocking out the PAH gene, (C) simulates the effective therapy for PKU disease by only decreasing the Phe in the medium and (D) simulates the therapy for PKU disease consisting of providing extra Tyr and decreasing Phe in the medium.

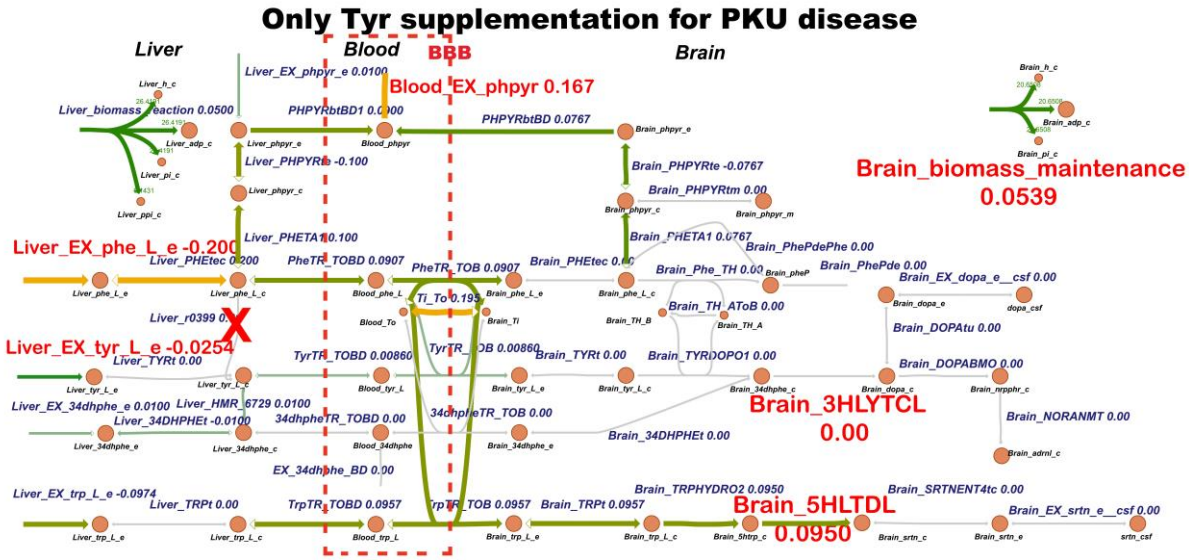

Figure S18: Effect of only adding extra Tyr (i.e. increasing the Tyr import flux) in the medium in the gene-expression-based model (Objective function = biomass production + dopamine production + serotonin production, all in the brain). Model used was 'Brain_liver_specific_plusthreeCompetitive'

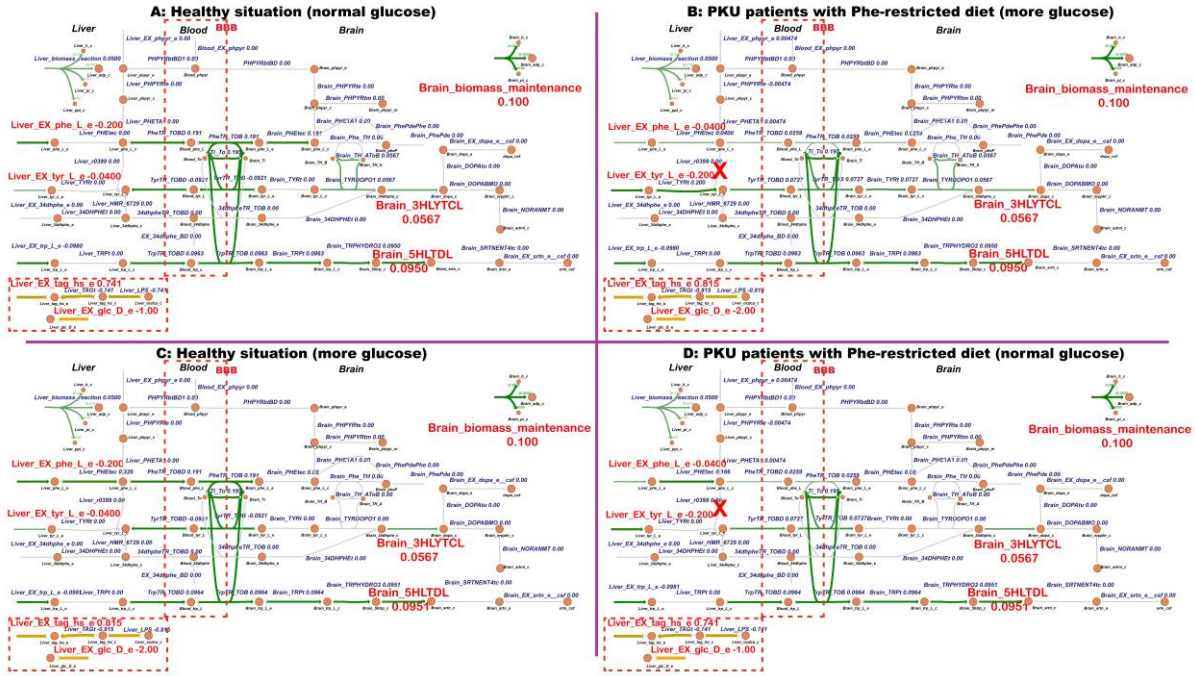

Figure S19: The triglyceride production in the gene-expression based tissue specific model for (A) healthy subjects with normal glucose uptake, (B) PKU patients with Phe-restricted diet (with high glucose uptake), (C) healthy persons with high glucose uptake and (D) PKU patients with Phe-restricted diet (with normal glucose uptake). The objective function was triglyceride production whilst the model was forced to satisfy what we defined as 'the essential needs' (a biomass reaction in brain of 0.1, a dopamine production of 0.057 and a serotonin production of 0.095). The 'Brain_liver_specific_plusthreeCompetitive' model above was used for these computations.

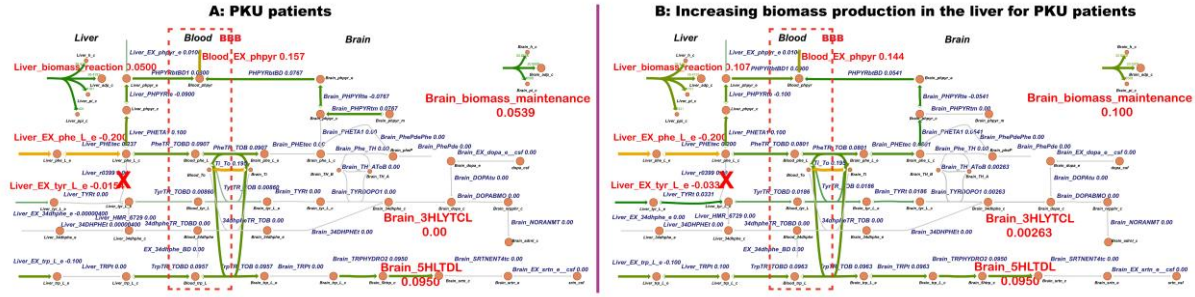

Figure S20: B versus A: The FBA result of increasing the biomass synthesis in the liver of PKU patients for the gene-expression-based model (i.e. simulated by increasing the upper bound for the 'biomass_reaction' in the liver) (Objective function: biomass reaction in brain (biomass_reaction_INB) + dopamine production (3HLYTCL_INB) + serotonin production (5HLTDL_INB)). The 'Brain_liver_specific_plusthreeCompetitive' model above was used for these computations.

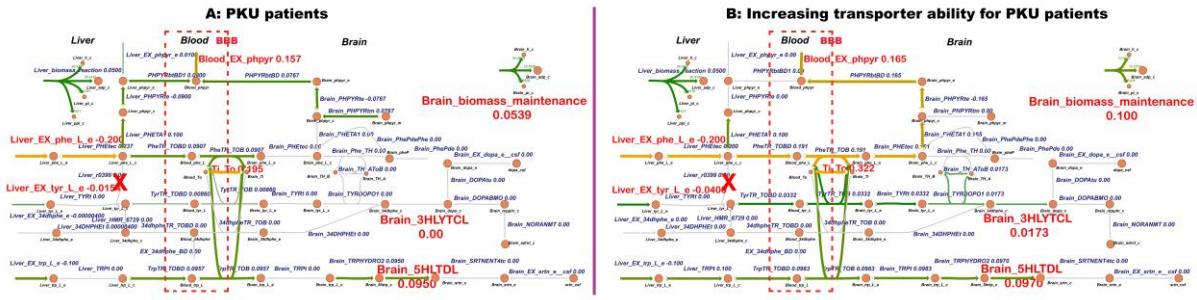

Figure S21: B versus A: The FBA result of increasing the transporter ability through the BBB for PKU patients, predicted using the gene-expression based model (increasing the upper bound for reaction 'Ti_To' from 0.195 to 0.322) (Objective function: biomass reaction in brain (biomass_reaction_INB) + dopamine production (3HLYTCL_INB) + serotonin production (5HLTDL_INB). The 'Brain_liver_specific_plusthreeCompetitive' model above was used for these computations.

Supplementary Tables

Table S1. New reactions in the 'Recon2.2plusthreeCompetitive' model that were absent from the Recon2.2 model. The transport reactions in the below are assumed to be reversible (i.e. [-1000,1000]) and exchange reaction are assumed to be irreversible (i.e. [0,1000]). All other reactions (e.g. PhePde_INB and Phe_TH_INB) were made irreversible ([0,1000]). Phe, Tyr and Trp used the same transporter (T1) from blood to brain. The other amino acids or O_2 or water used different, specific, transporters (T2-T8) from blood to brain. The transporters could also move back from brain to blood (i.e. reaction Ti1_To1 and Ti2_To2).

Transport reactions between liver and blood		Transport reactions between blood and brain	
Reaction ID	**Name**	**Reaction ID**	**Name**
GlcTR_TOBD	Transport reaction of Glucose from liver to blood	GlcTR_TOB	Transport reaction of Glucose from blood to brain
PheTR_TOBD	Transport reaction of Phenylalanine from liver to blood	PheTR_TOB	Transport reaction of Phenylalanine from blood to brain
PHPYRbtBD1	Transport reaction of Phenylpyruvate from liver to blood	PHPYRbtBD	Transport of Phenylpyruvate from brain to blood
TyrTR_TOBD	Transport reaction of Tyrosine from liver to blood	TyrTR_TOB	Transport reaction of Tyrosine from blood to brain
HisTR_TOBD	Transport reaction of Histidine from liver to blood	HisTR_TOB	Transport reaction of Histidine from blood to brain
IleTR_TOBD	Transport reaction of Isoleucine from liver to blood	IleTR_TOB	Transport reaction of Isoleucine from blood to brain
LeuTR_TOBD	Transport reaction of Leucine from liver to blood	LeuTR_TOB	Transport reaction of Leucine from blood to brain
MetTR_TOBD	Transport reaction of Methionine from liver to blood	MetTR_TOB	Transport reaction of Methionine from blood to brain
ThrTR_TOBD	Transport reaction of Threonine from liver to blood	ThrTR_TOB	Transport reaction of Threonine from blood to brain
TrpTR_TOBD	Transport reaction of Tryptophan from liver to blood	TrpTR_TOB	Transport reaction of Tryptophan from blood to brain
ValTR_TOBD	Transport reaction of Valine from liver to blood	ValTR_TOB	Transport reaction of valine from blood to brain
LysTR_TOBD	Transport reaction of Lysine from liver to blood	LysTR_TOB	Transport reaction of Lysine from blood to brain
AlaTR_TOBD	Transport reaction of Alanine from liver to blood	AlaTR_TOB	Transport reaction of Alanine from blood to brain
ArgTR_TOBD	Transport reaction of Arginine from liver to blood	ArgTR_TOB	Transport reaction of Arginine from blood to brain
AsnTR_TOBD	Transport reaction of Asparagine from liver to blood	AsnTR_TOB	Transport reaction of Asparagine from blood to brain
AspTR_TOBD	Transport reaction of Aspartic acid from liver to blood	AspTR_TOB	Transport reaction of Aspartic acid from blood to brain
CysTR_TOBD	Transport reaction of Cysteine from liver to blood	CysTR_TOB	Transport reaction of Cysteine from blood to brain
GluTR_TOBD	Transport reaction of Glutamic acid from liver to blood	GluTR_TOB	Transport reaction of Glutamic acid from blood to brain
GlnTR_TOBD	Transport reaction of Glutamate from liver to blood	GlnTR_TOB	Transport reaction of Glutamate from blood to brain
GlyTR_TOBD	Transport reaction of Glycine from liver to blood	GlyTR_TOB	Transport reaction of Glycine from blood to brain
ProTR_TOBD	Transport reaction of Proline from liver to blood	ProTR_TOB	Transport reaction of Proline from blood to brain
SerTR_TOBD	Transport reaction of Serine from liver to blood	SerTR_TOB	Transport reaction of Serine from blood to brain
O2TR_TOBD	Transport reaction of O_2 from liver to blood	O2TR_TOB	Transport reaction of O_2 from blood to brain
nh4TR_TOBD	Transport reaction of Ammonium from liver to blood	nh4TR_TOB	Transport reaction of Ammonium from blood to brain
piTR_TOBD	Transport reaction of Hydrogen phosphate from liver to blood	piTR_TOB	Transport reaction of Hydrogen phosphate from blood to brain
34dhpheTR_TOBD	Transport reaction of Levodopa from liver to blood	34dhpheTR_TOB	Transport reaction of Levodopa from blood to brain
acac_TOBD	Transport reaction of acetoacetate from liver to blood	acac_TOB	Transport reaction of acetoacetate from blood to brain

bhb_TOBD	Transport reaction of R)-3-hydroxybutyrate from liver to blood	bhb_TOB	Transport reaction of R)-3-hydroxybutyrate from blood to brain
co2_TOBD	Transport reaction of co2 from liver to blood	co2_TOB	Transport reaction of co2 from blood to brain
h_TOBD	Transport reaction of proton from liver to blood	h_TOB	Transport reaction of proton from blood to brain
h2o_TOBD	Transport reaction of water from liver to blood	h2o_TOB	Transport reaction of water from blood to brain
lac_D_TOBD	Transport reaction of (R)-lactate from liver to blood	lac_D_TOB	Transport reaction of (R)-lactate from blood to brain
lac_L_TOBD	Transport reaction of (S)-lactate from liver to blood	lac_L_TOB	Transport reaction of (S)-lactate from blood to brain
so4_TOBD	Transport reaction of sulfate from liver to blood	so4_TOB	Transport reaction of sulfate from blood to brain
urea_TOBD	Transport reaction of urea from liver to blood	urea_TOB	Transport reaction of urea from blood to brain
dopa_TOBD	Transport reaction of dopamine from liver to blood	dopa_TOB	Transport reaction of dopamine from blood to brain
adrnl_TOBD	Transport reaction of adrenaline from liver to blood	adrnl_TOB	Transport reaction of adrenaline from blood to brain
srtn_TOBD	Transport reaction of serotonin from liver to blood	srtn_TOB	Transport reaction of serotonin from blood to brain
Exchange reaction in the blood			
EX_phpyr_BD	Exchange reaction of Phenylpyruvate from blood	EX_34dhphhe_BD	Exchange reaction of levodopa in blood
EX_glc_D_BD	Exchange reaction of glucose from blood	EX_phe_L_BD	Exchange reaction of phenylalanine in blood
EX_tyr_L_BD	Exchange reaction of tyrosine from blood	EX_his_L_BD	Exchange reaction of histone from blood
EX_ile_L_BD	Exchange reaction of isoleucine from blood	EX_leu_L_BD	Exchange reaction of leucine from blood
EX_met_L_BD	Exchange reaction of methionine from blood	EX_thr_L_BD	Exchange reaction of threonine from blood
EX_trp_L_BD	Exchange reaction of tryptophan from blood	EX_val_L_BD	Exchange reaction of valine from blood
EX_lys_L_BD	Exchange reaction of lysine from blood	EX_ala_L_BD	Exchange reaction of alanine from blood
EX_arg_L_BD	Exchange reaction of arginine from blood	EX_asn_L_BD	Exchange reaction of asparagine from blood
EX_asp_L_BD	Exchange reaction of aspartic acid from blood	EX_cys_L_BD	Exchange reaction of cystine from blood
EX_glu_L_BD	Exchange reaction of glutamic acid from blood	EX_gln_L_BD	Exchange reaction of glutamate from blood
EX_gly_L_BD	Exchange reaction of glycine from blood	EX_pro_L_BD	Exchange reaction of proline from blood
EX_ser_L_BD	Exchange reaction of serine from blood	EX_o2_BD	Exchange reaction of oxygen from blood
EX_nh4_BD	Exchange reaction of ammonium from blood	EX_pi_BD	Exchange reaction of hydrogen phosphate from blood
EX_acac_BD	Exchange reaction of acetoacetate from blood	EX_bhb_BD	Exchange reaction of R)-3-hydroxybutyrate from blood
EX_co2_BD	Exchange reaction of co2 from blood	EX_h_BD	Exchange reaction of proton from blood
EX_h2o_BD	Exchange reaction of water from blood	EX_lac_D_BD	Exchange reaction of (R)-lactate from blood
EX_lac_L_BD	Exchange reaction of (S)-lactate from blood	EX_so4_BD	Exchange reaction of sulfate from blood
EX_urea_BD	Exchange reaction of urea from blood	EX_dopa_BD	Exchange reaction of dopamine from blood
EX_adrnl_BD	Exchange reaction of adrenaline from blood	EX_srtn_BD	Exchange reaction of serotonin from blood
Other Reactions			
Ti1_To1	Transporter1 moving back to blood	Ti2_To2	Transporter2 moving back to blood
Ti3_To3	Transporter3 moving back to blood	Ti4_To4	Transporter4 moving back to blood
Ti5_To5	Transporter5 moving back to blood	Ti6_To6	Transporter6 moving back to blood
Ti7_To7	Transporter7 moving back to blood	Ti8_To8	Transporter8 moving back to blood
Phe_TH_INB	Phenylalanine conversion to 'product' catalyzed by tyrosine hydroxylase in brain	PhePde_INB	Phenylalanine 'product' degradation in brain
TYRDOPO1_INB	Levodopa production in brain	EX_TH_B_INB	Tyrosine hydroxylase produced reaction
TH_AToB_INB	Tyrosine hydroxylase dissociated reaction	PhePdePhe_INB	Phenylalanine product degradation to Phe in brain

Table S2. The 'simple medium composition' used here (and in Mondeel et al., 2018) for the Recon2.2plusthreeCompetitive model. This was effected as follows: In the original Recon2.2[24], which we had downloaded from 'BioModels', we changed the negative bound for the exchange reactions of any compound mentioned below under 'Metabolites' to minus the corresponding value indicated under 'Composition'. The negative (import) bounds for all other substances were set to zero. Pi refers to hydrogen phosphate HPO_4^{2-}. Numbers >=1000 represent vast excess. All medium components could only be taken up by the liver, with the exception of O_2 which was taken up by the blood.

Metabolites	Composition	Metabolites	Composition	Metabolites	Composition
Glucose	1	Lys	0.1	Pi	999999
H^+	999999	Met	0.1	SO_4^{2-}	999999
H_2O	999999	NH_4^+	999999	Thr	0.1
His	0.1	O_2	1000	Trp	0.1
Ile	0.1	Phe	0.1	Val	0.1
Leu	0.1	Tyr	0.04	CO_2	999999

Table S5. Blood biomarker prediction for PKU disease (PAH gene). All compounds with an exchange reaction and with positive upper bound value were considered in terms of biomarker potential. There were no exchange reactions from the brain. All these exchange reactions in the liver had an upper (export) bound of '0' and the same lower bound as in Recon2.2plusthreeCompetitive (Table S2), and all exchange reactions in the blood had a lower bound of '0' and an unlimited upper bound representing secretion by the kidneys. The flux variability in these exchange reactions is indicated between [] both for the healthy model ('WT') and the PAH deleted model ('Mutant'). The PAH deletion removed both the 'r0399' and the 'PHETHPTOX2' reaction from the liver When the mutant had a lower maximum exchange flux than the WT the prediction was that the metabolite should be reduced upon PAH deletion. When it had a higher maximum flux, the prediction was 'elevated'. 'Score' was calculated as the fractional change of that maximum flux [Shlomi et al]. Only metabolites with a score in excess of '0.1' are shown here. Phenylalanine exchange from the blood was not normally present, but only implemented when asking whether this Phe itself could be a biomarker.

ID	Name	Prediction	WT	Mutant	Score
phpyr_BD	keto-phenylpyruvate	H.C. Elevated	[0.0, 0.1]	[0.163, 0.163]	1
dopa_BD	dopaminium(1+)	Reduced	[0.0, 0.177]	[0.0, 0.017]	0.90396
adrnl_BD	adrenaline	Reduced	[0.0, 0.177]	[0.0, 0.017]	0.90396
tyr_L_BD	L-tyrosine	Reduced	[0.0, 0.12]	[0.0, 0.017]	0.85833
34dhphe_BD	L-dopa	Reduced	[0.0, 0.12]	[0.0, 0.017]	0.85833
lys_L_BD	L-lysine	Elevated	[0.0, 0.011]	[0.0, 0.016]	0.3125
leu_L_BD	L-leucine	Elevated	[0.0, 0.018]	[0.0, 0.022]	0.18182
Adding virtual exchange reaction for Phe in the blood to check whether it is a biomarker					
Phe_L_BD	L-phenylalanine	Elevated	[0.0, 0.12]	[0.02, 0.161]	1

Table S5A. 'Hidden-biomarker' prediction for PKU disease (PAH gene) with the 'Recon2.2plusthreeCompetitive' model, for 17 exemplary compounds in the brain. Each of these 'hidden' compounds (called hidden because they lacked an exchange reaction in the model) was examined individually for biomarker potential by adding an exchange reaction for it and comparing the possible flux range through that reaction between the healthy ('WT') and the PAH gene deleted version of the model ('Mutant'). The PAH deletion removed both the 'r0399' and the 'PHETHPTOX2' reaction from the liver. The FVA results of metabolites in these virtual exchange reactions before and after knocking out the PAH gene are compared. Only metabolites with a score in excess of 0.1 are shown as 'Hidden Biomarkers' here; the others are referred to as 'Hidden Non-Biomarkers'.

Hidden Biomarkers					
ID	**Name**	**Prediction**	**WT**	**Mutant**	**Score**
2hyoxplac_INB_e	(2-hydroxyphenyl) acetic acid (brain)	Elevated	[0.0, 0.0]	[0.0, 0.063]	1
pheP_INB_e	phenylalanine product with tyrosine hydroxylase (brain)	Elevated	[0.0, 0.0]	[0.0, 0.063]	1
peamn_INB_e	2-phenylethanaminium (brain)	Elevated	[0.0, 0.0]	[0.0, 0.063]	1
pacald_INB_e	Phenylacetaldehyde (brain)	Elevated	[0.0, 0.0]	[0.0, 0.063]	1
pac_INB_e	Phenylacetate (brain)	Elevated	[0.0, 0.0]	[0.0, 0.063]	1
34dhmald_INB_e	3,4-dihydroxymandelaldehyde (brain)	Reduced	[0.0, 0.177]	[0.0, 0.017]	0.9
Hidden Non-Biomarkers					
thbpt_INB_e	5,6,7,8-tetrahydrobiopterin (BH4) (brain)				
Nacsertn_INB_e	N-acetyl serotonin (brain)				
melatn_INB_e	Melatonin (brain)				
6hoxmelatn_INB_e	6-Hydroxymelatonin (brain)				

fna5moxam_INB_e	Formyl-N-acetyl-5-methoxykynurenamine (brain)				
dhbpt_INB_e	6,7-dihydrobiopterin (BH2) (brain)				
thbpt4acam_INB_e	4a-hydroxytetrahydrobiopterin (brain)				
phaccoa_INB_e	phenylacetyl-CoA (4-) (brain)				
tym_INB_e	Tirocinium (brain)				
3ityr_L_INB_e	3-iodo-L-tyrosine (brain)				
iodine_INB_e	Diiodine (brain)				

Table S6. Biomarker prediction for PKU disease (reaction 'r0399' and 'PHETHPTOX2' being deleted) by using the single compartment Recon2 model. All exchange reactions had a lower bound of '-1' and an unlimited upper bound. The objective function for the model is biomass production, and we compared the biomarkers when the model reached minimal percentage of optimum of objective function of 100%, 90%, 50% and 0. In italics we show the biomarkers predicted by Thiele et al (2020).

Minimal percentage of the optimum of the objective function=100%					
No biomarkers found					
Minimal percentage of the optimum of the objective function=90%					
ID	**Name**	**Prediction**	**WT**	**Mutant**	**Score**
34hpp_e	3-(4-hydroxyphenyl) pyruvate	Reduced	[-1.0, 257.263]	[-1.0, 139.815]	0.46
tyr_L_e	L-tyrosine	Reduced	[-1.0, 257.263]	[-1.0, 139.815]	0.46
34dhphe_e	L-dopa	Reduced	[-1.0, 258.263]	[-1.0, 140.815]	0.46
tymsf_e	Tyramine O-sulfate	Reduced	[0.0, 258.263]	[0.0, 140.815]	0.46
4hphac_e	4-hydroxyphenylacetate	Reduced	[0.0, 258.263]	[0.0, 140.815]	0.46
dopa_e	dopaminium(1+)	Reduced	[-1.0, 259.263]	[-1.0, 141.815]	0.45
nrpphr_e	Norepinephrine	Reduced	[-1.0, 260.263]	[-1.0, 142.815]	0.45
dopasf_e	dopamine 3-O-sulfate	Reduced	[0.0, 260.263]	[0.0, 142.815]	0.45
34dhoxpeg_e	3,4-Dihydroxyphenylethyleneglycol	Reduced	[0.0, 261.263]	[0.0, 143.815]	0.45
adrnl_e	Adrenaline	Reduced	[0.0, 261.263]	[0.0, 143.815]	0.45
nrpphrsf_e	Sulfate derivative of norepinephrine	Reduced	[0.0, 261.263]	[0.0, 143.815]	0.45
mepi_e	Metanephrine	Reduced	[0.0, 261.263]	[0.0, 143.815]	0.45
Minimal percentage of the optimum of the objective function=50%					
ID	**Name**	**Prediction**	**WT**	**Mutant**	**Score**
tyr_L_e	L-tyrosine	Reduced	[-1.0, 371.813]	[-1.0, 183.453]	0.51
34hpp_e	3-(4-hydroxyphenyl) pyruvate	Reduced	[-1.0, 371.813]	[-1.0, 183.453]	0.51
4hphac_e	4-hydroxyphenylacetate	Reduced	[0.0, 372.813]	[0.0, 184.453]	0.51
34dhphe_e	L-dopa	Reduced	[-1.0, 372.813]	[-1.0, 184.453]	0.51
tymsf_e	Tyramine O-sulfate	Reduced	[0.0, 372.813]	[0.0, 184.453]	0.51
dopa_e	dopaminium(1+)	Reduced	[-1.0, 373.813]	[-1.0, 185.453]	0.5
nrpphr_e	Norepinephrine	Reduced	[-1.0, 374.813]	[-1.0, 186.453]	0.5
dopasf_e	dopamine 3-O-sulfate	Reduced	[0.0, 374.813]	[0.0, 186.453]	0.5
34dhoxpeg_e	3,4-Dihydroxyphenylethyleneglycol	Reduced	[0.0, 375.813]	[0.0, 187.453]	0.5
mepi_e	Metanephrine	Reduced	[0.0, 375.813]	[0.0, 187.453]	0.5
nrpphrsf_e	Sulfate derivative of norepinephrine	Reduced	[0.0, 375.813]	[0.0, 187.453]	0.5
adrnl_e	Adrenaline	Reduced	[0.0, 375.813]	[0.0, 187.453]	0.5
Minimal percentage of the optimum of the objective function=0					
ID	**Name**	**Prediction**	**WT**	**Mutant**	**Score**
4hpp_e	3-(4-hydroxyphenyl) pyruvate	Reduced	[-1.0, 515.0]	[-1.0, 238.0]	0.54
tyr_L_e	L-tyrosine	Reduced	[-1.0, 515.0]	[-1.0, 238.0]	0.54
4hphac_e	4-hydroxyphenylacetate	Reduced	[0.0, 516.0]	[0.0, 239.0]	0.54
34dhphe_e	L-dopa	Reduced	[-1.0, 516.0]	[-1.0, 239.0]	0.54
dopa_e	dopaminium(1+)	Reduced	[-1.0, 517.0]	[-1.0, 240.0]	0.54
nrpphr_e	Norepinephrine	Reduced	[-1.0, 518.0]	[-1.0, 241.0]	0.54
34dhoxpeg_e	3,4-Dihydroxyphenylethyleneglycol	Reduced	[0.0, 519.0]	[0.0, 242.0]	0.53
adrnl_e	Adrenaline	Reduced	[0.0, 519.0]	[0.0, 242.0]	0.53
mepi_e	Metanephrine	Reduced	[0.0, 508.0]	[0.0, 242.0]	0.52
tymsf_e	Tyramine O-sulfate	Reduced	[0.0, 501.0]	[0.0, 239.0]	0.52
dopasf_e	dopamine 3-O-sulfate	Reduced	[0.0, 501.0]	[0.0, 241.0]	0.52
nrpphrsf_e	Sulfate derivative of norepinephrine	Reduced	[0.0, 501.0]	[0.0, 242.0]	0.52
Biomarkers predicted by Thiele et al (2020)					
phpyr_BD	*keto-phenylpyruvate*				

Phe_L_BD	*L-phenylalanine*				
2hyoxplac_INB_e	*(2-hydroxyphenyl) acetic acid*				

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