Early diagnosis of inborn errors of metabolism is commonly performed through biofluid metabolomics, which detects specific metabolic biomarkers whose concentration is altered due to genomic mutations. The identification of new biomarkers is of major importance to biomedical research and is usually performed through data mining of metabolomic data. After the recent publication of the genome-scale network model of human metabolism, we present a novel computational approach for systematically predicting metabolic biomarkers in stoichiometric metabolic models. Applying the method to predict biomarkers for disruptions of red-blood cell metabolism demonstrates a marked correlation with altered metabolic concentrations inferred through kinetic model simulations. Applying the method to the genome-scale human model reveals a set of 233 metabolites whose concentration is predicted to be either elevated or reduced as a result of 176 possible dysfunctional enzymes. The method’s predictions are shown to significantly correlate with known disease biomarkers and to predict many novel potential biomarkers. Using this method to prioritize metabolite measurement experiments to identify new biomarkers can provide an order of a 10-fold increase in biomarker detection performance.

Introduction

The study of genetic metabolic disorders originated in the early 1900s with Sir Archibald Garrod’s discovery of the first inborn errors of metabolism (IEM), alkaptonuria, pentosuria, cystinuria, and albinism (Vangala and Tonelli, 2007). IEM are caused by alterations of specific metabolic reactions and a few hundreds of IEM affecting about 1 in every 5000 born babies are currently characterized (Lanpher et al., 2006). Fortunately, recent advances in metabolomic approaches enable an expanded newborn screening that improves early diagnosis and treatment in numerous IEM disorders (Lanpher et al., 2006).

Metabolomics, the study of the complete repertoire of small molecules in cells, tissues and biological fluids, represents a major and rapidly evolving research field in systems biology. It has been fueled by the development of experimental platforms such as gas chromatography and liquid chromatography-based mass spectrometry that are capable of accurately measuring hundreds of small molecules in biological samples (Kaddurah-Daouk et al., 2008). These methods promise to substantially advance our understanding of disease pathophysiology and advance the discovery of new diagnostic biomarkers for disease. Such metabolic biomarkers denote sets of metabolites that show a consistent change in concentration during a disease state and are hence effective diagnostic means. Metabolomics offers several advantages over genomics and proteomics as a tool for diagnosing and understanding disease (Vangala and Tonelli, 2007): (1) Metabolic
Prediction of human metabolic biomarkers
T Shlomi et al

Biomarkers can be measured noninvasively in biofluids such as plasma, urine and feces in a rather straightforward manner (Seegmiller, 1968). (2) Changes in metabolite levels reflect the actual metabolic state of a tissue and its histology, translating the genotype and environmental factors into the phenotype (Nicholson et al., 2002) (Urbanczyk-Wochniak et al., 2003). (3) There is a relatively small number of biomarkers (~2500–3000). Several IEM have already been characterized through the identification of clinical metabolic biomarkers that can explain the pathological phenotype (Ito et al., 2000). However, with the expected surge in the scope and quality of metabolomic measurements, metabolomics is destined to play an even more central role in the near future as an efficient diagnostic tool and as a safety evaluator of drug candidates.

The recent publication of the first Human metabolic network model (Duarte et al., 2007), along with the detailed documentation of all known IEM in the OMIM database (McKusick, 2007), have given us an opportunity to systematically predict potential metabolic disease biomarkers on a large scale. Herewith, we propose a new computational approach that predicts, for each metabolic gene, a set of metabolites that are expected to show a concentration change in biofluids after its knockout. The method is based on the constraint-based modeling (CBM) approach, which is commonly used to predict metabolic phenotypes in microorganisms (Price et al., 2004) and specifically the effects of gene knockouts (Segre et al., 2002; Stelling et al., 2002; Shlomi et al., 2005). Recently, CBM has been used to predict human tissue-specific metabolism (Duarte et al., 2007; Shlomi et al., 2008). Our approach differs from earlier attempts to identify disease biomarkers, which mostly use data mining techniques that analyze metabolomics data taken from healthy and diseased subjects (Wagner et al., 2004; Yang et al., 2004; Kenny et al., 2005). As it is model based, the current approach permits the prediction of large sets of diagnostic biomarker patterns for many disorders, laying down a computational parallel to the upcoming advances in metabolomic measurements in biofluids. As a first validation of our method, we applied it to predict changes in metabolic concentrations due to dysfunctional enzymes in red-blood cell (RBC) metabolism, whose dynamic behavior can be reliably simulated through a kinetic model (Jamshidi et al., 2001). Then, the method is applied to the genome-scale human metabolic network model of Duarte et al., and its performance is comprehensively evaluated based on various sets of known biomarker extracted from different databases.

Results and discussion

A constraint-based approach for predicting metabolic biomarkers

We present a new computational approach for systematically predicting the pattern of metabolic biomarkers characterizing each metabolic disorder whose causative gene is included in the human metabolic network model (Duarte et al., 2007). Let a boundary metabolite denote a metabolite that is known to be taken-up or secreted between the intracellular and extracellular compartments (as indicated in the network model). Let an exchange interval denote a possible range of uptake and secretion fluxes of a given boundary exchange interval. For each metabolic disorder and each boundary metabolite, we predict its exchange interval between human tissues and biofluids, for both healthy and disease cases (Materials and methods). This exchange interval is computed through a CBM method called flux variability analysis (FVA) (Mahadevan and Schilling, 2003), which accounts for the entire space of feasible flux states that satisfy mass-balance stoichiometric constraints and reaction directionality constraints (embedded in the model of (Duarte et al., 2007)). For the healthy case, the exchange interval is computed while the reactions affected by the disease are constrained to be active, whereas for the disease case, they are constrained to be inactive. By comparing the predicted exchange interval between the healthy state and the disease state for each boundary metabolite, one can determine whether the pertaining boundary metabolite concentration in biofluids (termed biomarker) is expected to be elevated, reduced or unchanged (see Materials and methods). If the predicted changes are marked such that there is no overlap between the exchange intervals of the healthy case and the disease case, the predicted biomarker change is considered to be highly confident.

An illustrative example of the predicted biomarker changes’ ranges and their underlying rationale for the healthy state and some disease state is depicted in Figures 1A and B. The predicted exchange intervals of metabolite M1 (M2) suggest that its extracellular concentration is elevated (reduced) in the disease case. The disjoint exchange intervals obtained for the healthy case and the disease case for both M1 and M2 render these predictions as highly confident. The exchange intervals of metabolite M6 (M4) suggest that their extracellular concentrations are elevated (reduced) in the disease case. Examining, for example, the exchange interval of metabolite M6 shows that in the healthy case, M6 can be either taken-up from biofluids or secreted in a lower rate (as some of it is required in the healthy state; Supplementary Figure 1). In the disease case, M6 (synthesized through M5) can only be secreted to biofluids. It should be noted that mass-balance stoichiometric constraints that play an important role in determining the exchange intervals of different metabolites and are accounted for by the CBM method (and as will be shown, play an important role in determining biomarker changes in addition to the network topology) are not depicted in this kind of illustration.

Validating the biomarker prediction method through a small-scale kinetic model of RBC metabolism

As a first validation of our method, we applied it to predict metabolic biomarkers for enzyme deficiencies in human erythrocytes, for which a detailed kinetic model (Jamshidi et al., 2001) is readily available to simulate the dynamic metabolic behavior after enzymatic perturbations. This kinetic model consists of four basic classical pathways: glycolysis, the pentose pathway, adenosine nucleotide metabolism, and the Rapoport-Leubering shunt, accounting for 43 metabolites, 43 internal reactions, and 12 primary exchange reactions. We applied this model to predict changes in extracellular metabolite concentrations after a disruption to 43 enzyme-
catalyzed reactions in the model (i.e. simulating changes in the steady-state behavior by iteratively solving the set of differential equations specified in the model; Jamshidi et al., 2001; see Materials and methods). These simulations resulted in a set of 156 metabolic biomarkers whose extracellular concentration is either elevated or reduced in a perturbed steady-state behavior.

Earlier studies have shown that CBM of RBC metabolism correctly captures various aspects of the metabolic behavior simulated through a kinetic model (Wiback and Palsson, 2002; Durmus Tekir et al., 2006). Applying our constraint-based method for the RBC model to predict changes in extracellular metabolites after the knockouts of model reactions resulted in a set of 85 biomarker predictions (see Materials and methods). These biomarker predictions are significantly correlated with the kinetic simulation results (hypergeometric P-value < \(7 \times 10^{-31}\), comparing the predicted accuracy with a random model), obtaining a precision of 0.73 (fraction of the predicted biomarkers that are correct) and recall of 0.40 (fraction of the biomarkers that are correctly predicted; Table I). This result testifies to the ability of our method to correctly identify alterations in extracellular metabolite concentrations, relying solely on reaction stoichiometry and directionality data.

![Figure 1](https://example.com/figure1.png)

**Table 1** Prediction accuracy of the biomarker prediction method based on a comparison with predictions obtained through kinetic model of red-blood cell metabolism, and based on comparisons with various experimental datasets

| Prediction Method          | Precision | Recall | P-value     |
|----------------------------|-----------|--------|-------------|
| RBC Kinetic Model          | 0.73      | 0.40   | \(<7 \times 10^{-31}\) |
| OMIM: Automatic            | 0.37      | 0.27   | \(<7 \times 10^{-12}\) |
| OMIM: Manual               | 0.76      | 0.56   | \(<4 \times 10^{-13}\) |
| Ramedis/HMDB               | 0.41      | 0.1    | \(<5 \times 10^{-5}\) |

A detailed comparison of the predictions versus the known biomarkers is available in Supplementary Dataset 1.

**Large-scale prediction and validation of biomarkers for human metabolic disorders**

We applied our method to the human metabolic network model (Duarte et al., 2007) to predict biomarker changes for 304 metabolic disorders (documented in the OMIM database) whose causative genes are included in the model (see Materials and methods). The analysis resulted in a total of 3912 predictions of biomarker’s changes involving 233 boundary metabolites (whose concentration is predicted to change in at least a single disease), and 176 diseases (for which at least a single biomarker change is predicted). Out of all
biomarker alteration predictions, 19% are with high
confidence. A high fraction of the disorders (42 %) are predicted to
have very few biomarker changes (< 6), whereas up to 61 % of
the disorders are predicted to have < 10 biomarkers (Figure 1C).

The various disorders tend to have different sets of
biomarkers (e.g. only in a very few cases, the same set of
biomarkers correspond to more than three diseases; Figure 1D), suggesting that large-scale biofluids’ metabo-
lonics may be effectively used for the diagnosis of metabolic
disorders. Notably, the majority of the predicted biomarkers
(129 metabolites out of 175 for which this information is
available) are known to be present in the blood and urine and
are hence readily available media for metabolomic analysis
(Human Metabolome Database (HMDB); Wishart et al., 2007;
Supplementary Figure 2).

To systematically validate the predictions, we extracted
biomarker data for all metabolic disorders documented in the
OMIM database, whose causing genes are included in the
model of Duarte et al (by automatic parsing of disease
description texts in the OMIM database; see Materials and
methods). Comparing this dataset with our predictions
showed a highly significant correlation (hypergeometric
P-value < 7 × 10⁻¹²), though with precision and recall levels
lower than those described above versus the kinetic RBC
model (Table I). Still, this correlation is quite remarkable,
considering the erroneous nature of the simple text-mining
method that underlies this validation dataset.

To derive a more refined biomarker dataset for validation,
we manually extracted biomarker data from the OMIM
database for a set of 17 inborn errors of amino-acid
metabolism (see Materials and methods). This manual

curation process permits a finer tuned resolution of problems
arising in the validation data, for example, from differences in
metabolite naming conventions. Furthermore, it allows one to
extract data on the precise specific enzymatic reactions that are
affected in each metabolic disorder, in cases where mutations
may disrupt the activity of multifunctional genes. A compar-
ison of this dataset to the model predictions again demon-
strated a fairly accurate level of prediction (hypergeometric
P-value < 4 × 10⁻¹³; precision = 0.76, recall = 0.56; Figure 2),
close to that shown above versus the kinetic RBC model
(Table I).

To further validate our prediction method, we extracted
biomarker data for a set of 29 rare metabolic disorders from the
Rare Metabolic Disease database (Ramedis; Töpel et al., 2006),
recording clinical measurements of metabolite levels in
biofluids, and from HMDB (Wishart et al., 2007; see Materials
and methods). Comparing the set of predicted biomarkers
alterations to this clinical dataset exhibited a highly statisti-
cally significant overlap (hypergeometric P-value < 5 × 10⁻⁸),
with moderate precision (0.41) but rather low recall (0.1)
(Table I). This lower accuracy level may result in part from the
lower quality of the pertaining clinical data; the latter is prone
to the influence of several nondisorder-specific factors (e.g. the
medical treatment the patient was subject to, or his nutritional
state). Indeed, cross-referencing these clinical biomarkers
with those reported by OMIM shows a rather low overlap
between the two different kinds of data sources (50% of the
biomarker-diseases associations marked in OMIM are found in

![Figure 2](image-url)

**Figure 2** Prediction of amino-acid biomarkers for a set of amino-acid metabolic disorders. Rows represent metabolic disorders and columns represent amino acids. The causative gene’s name is indicated on the left. Blue and red entries represent biomarkers that are predicted by our method to be elevated or reduced, respectively. Table entries marked in ‘+‘ or ‘-‘ represent elevation or reduction in the metabolite’s concentration in biofluids according to OMIM, respectively.
Ramedis-HMDB, but only 19% of the associations marked in Ramedis-HMDB are found in OMIM). Notably, the agreement between the different pertaining databases is of the order of their agreement with the model’s predictions.

**In-depth inspection of candidate biomarker predictions**

To explore the added value of our biomarker prediction method over naïve visual inspection of the network topology, we manually inspected the corresponding network regions affected by errors in amino-acid metabolism. We found that although in a few cases it might have been possible to correctly predict biomarkers just by observing the perturbed pathway in a topological map representation of the human metabolic network, in other cases such a simple topologically based inference of a biomarker alteration would fail and lead to false predictions. Thus, indeed, a method, accounting for the network dependencies between pathways along with stoichiometry and reaction directionality constraints is of value. For instance, in methionine adenosyltransferase deficiency (OMIM: 250850), the potential elevation of methionine in biofluids can be predicted in a straightforward manner as the only reaction that catabolizes methionine is inactivated in this disorder. However, in the cases of homocystinuria, hypermethioninemia, and tyrosinemia that we discuss next, the network topology alone fails to identify the correct biomarkers.

An example of a correctly predicted biomarker that is difficult to infer simply by observing the network topology occurs in the case of homocystinuria, caused by the deficient activity of Cystathionine β-synthase (CBS; E.C 4.2.1.22; converting homocysteine and serine to cysteine; Figure 3). In the healthy case, when CBS is functional, methionine is taken-up from biofluids and is eventually converted to cysteine by series of enzymes that includes CBS. Our method predicts that the biofluids’ concentration of methionine is elevated (with high confidence) in homocystinuria as reported in OMIM, and that the concentration of cysteine is reduced in the extracellular as reported in (Lee and Briddon, 2007) (Figure 2). In this case, inferring the elevated extracellular concentration of methionine simply based on the network topology is impossible. This is because of the existence of an alternative cyclic pathway that metabolizes methionine in homocystinuria (the methionine salvage pathway; Cellarier et al, 2003), but in fact, cannot change the methionine extracellular concentration due to mass-balance constraints. A similar scenario pertains to the predicted elevated extracellular concentration of methionine in hypermethioninemia caused by S-adenosylhomocysteine hydrolase deficiency (AHCH; E.C 3.3.1.1; OMIM: 180960; Figure 3). Overall, although it is indeed difficult to make such predictions a priori by inspecting the network topology, such inspections can be telling and informative a posteriori (when pointed to by the stoichiometric analysis). Other interesting examples are the cases of tyrosinemia type I, type III, and Alkaptonuria (OMIM 276700, 276710, 203500, respectively), each caused by the dysfunctional behavior of one out of five tyrosine degradation pathways. A simple observation of the network topology may suggest that the existence of several alternative tyrosine

---

**Figure 3**  (A) A subnetwork that illustrates the effect of homocystinuria on the metabolism and transport of methionine. Circular nodes represent metabolites and edges represent biochemical reactions. For simplicity, only abbreviations of metabolite names and enzyme E.C (Enzyme Commission) numbers are specified (explicit names are given in Supplementary Table 1). Metabolites marked in green participate in other reactions that are not presented here for simplicity. Homocystinuria is caused by a dysfunctional CBS, and hypermethioninemia is caused by dysfunctional AHCH. (B) Prediction of concentration changes of the boundary metabolite methionine in homocystinuria and S-adenosylhomocysteine hydrolase deficiency based on interval comparison of its exchange reaction’s flux. In both cases, inferring the elevated concentration of methionine simply based on the network topology is impossible, due to the cyclic methionine salvage pathway (involving reaction 4.1.1.50), which (though topologically plausible) cannot affect methionine concentration due to mass-balance constraints.
degradation pathways may suffice to compensate for the inactivity of a single degradation pathway, and keep the pertaining metabolites’ extracellular concentrations at bay (Supplementary Figure 3). However, it turns out that due to mass-balance stoichiometric considerations the network cannot compensate for the inactivity of one degradation pathway by routing more substrates to the alternative active pathways, and, indeed, the elevated extracellular concentrations of tyrosine are correctly predicted by our method in all three diseases (Figure 2).

Although our method successfully identifies a large set of the known biomarkers in a statistically significant manner, it failed to identify a considerable number of others. Some of the false predictions are expected to result from incompleteness of the metabolic network as well as several simplifying assumptions that underlie our computational method and enable the large-scale analysis of a network with thousands of reactions. Specifically, our method assumes a steady-state metabolic behavior (due to the lack of global enzyme kinetic data), and lacks regulatory constraints, which obviously have substantial influence on the activation of alternative pathways under different physiological conditions (but are still largely missing for the human case). In fact, the lack of cell type- and tissue-specific regulatory constraints restricts the analysis to a system that integrates the metabolic behavior of different tissues. The latter prevents the prediction of cross-tissue mechanisms that affect metabolite biofluid concentration (e.g. a metabolite secreted in one tissue and taken-up by another). However, although the method relies on several simplifying assumptions, its predicted biomarkers are significantly correlated with all tested validation datasets, with the precision and recall varying between 0.37–0.76 and 0.1–0.56, respectively, depending on the quality of the dataset. The probability that a biomarker prediction made by our method would turn out to be correct is between 6–15.8 times higher than random, across the various validation datasets (based on random generation of biomarker sets and a comparison of their prediction accuracy with our method). This suggests that using our method to prioritize metabolite measurement experiments can provide an order of a 10-fold increase in biomarker detection performance. This result is remarkably encouraging in face of the obvious model simplifications discussed above.

An in-depth investigation of the false predictions can be of significant value for further improving the metabolic network model and the analysis methods. For example, in the case of methylmalonate semialdeyde dehydrogenase deficiency (OMIM 603178), in which a reaction (E.C 1.2.1.27) in the valine and pyrimidine catabolic pathways is dysfunctional, our method falsely predicts the potential elevation of valine in biofluids instead of the elevation of its catabolic product 3-hydroxyisobutyric acid, as well as 3-hydroxyproionic acid and β-alanine. An inspection of the underlying network topology reveals that this false prediction results from missing membrane transporters for these products and their related derivatives, which prevent their exchange with surrounding biofluids and hence indirectly limits the possible uptake of their upstream substrate, valine (Supplementary Figure 4).

Incorporating a putative transporter of 3-hydroxyisobutyric acid from mitochondria to cytoplasm and from there to the extracellular environment in the model, enables our method to correctly predict the increased secretion rate of 3-hydroxyisobutyric acid in this disorder (instead of the increased concentration of its upstream substrate, valine). Similarly, the inclusion of a transporter for 3-hydroxyisobutyric acid and a mitochondrial transporter for its derivative, malonate semialdeyde, leads to a prediction of decreased uptake rate of 3-hydroxyisobutyric acid in these disorders (reflecting an increased biofluid concentration), and to correctly predicting the elevated concentration of β-alanine. These results suggest a future application of the biomarker prediction method for automatically identifying missing reactions in the model, in line with a previous approach for refining genome annotation (Reed et al, 2006).

In other cases, the lack of regulatory constraints leads to false predictions. For example, the model fails to predict the elevation in extracellular concentration of arginine in arginemia (OMIM 207800). The latter elevation is caused by a dysfunctional type I arginase (ARG1; E.C 3.5.3.1), which disrupts the conversion of arginine to urea and ornithine. This prediction failure arises from the existence of several additional alternative pathways that catabolize arginine (e.g. converting it either to N-hydroxyarginine, guanidinoacetate, and ornithine, or transporting it to the mitochondria) and maintain the same predicted uptake rate of arginine when ARG1 is dysfunctional. In reality, arginine catabolism through the mitochondria (through the type II arginase ARG2) is likely not to be able to fully compensate for the loss of ARG1 due to the low expression level of ARG2 compared with that of ARG1 in the liver (Levillain et al, 2005; Cline et al, 2007). Specifically, these erroneous predictions are probably caused by the current lack of tissue-specific regulatory constraints. Such prediction errors may be corrected by incorporating regulatory constraints within the model (Covert et al, 2004), or by incorporating tissue-specific expression data (Akeson et al, 2004; Duarte et al, 2007; Shlomi et al, 2008).

Although this paper focused on predicting metabolic biomarkers for known IEM, we additionally applied our approach to predict biomarkers for the knockouts of all other genes present in the metabolic model, that is, those that are not known to cause metabolic disorders but some may potentially be discovered to do so in the future. This analysis, covering an additional set of 872 genes from the model of Duarte et al, resulted in a total set of 9567 biomarkers alterations, which are available for inspection and future validation as Supplementary Dataset 1. To provide means for further studying the mechanisms by which a biomarker’s extracellular concentration is altered, we created network visualizations (through the Cytoscape tool (Cline et al, 2007)) of the metabolic alterations resulting from the knockout of each gene in the network. These are available for download from the supplemental website: www.cs.tau.ac.il/~shlomito/biomarkers.

In summary, this study presents a generic approach for the large-scale prediction of specific biomarkers that are elevated or reduced in biofluids. Future work should aim at extending the model to include additional metabolic pathways, for example, by integrating it with other large-scale networks such as the Edinburgh human metabolic network reconstruction (Ma et al, 2007). On the long run, an integrated human metabolic-regulatory reconstruction in the lines of that of...
(Covert et al., 2004) will further improve the predictions in cases where the predicted activation of alternative pathways is in discordance with their real biological activity. This will additionally require the usage of computational methods for solving such integrated models (Shlomi et al., 2007). Building upon the basic approach presented here, these approaches may further advance the search for reliable model-driven predictions of metabolic biomarker alterations on a genomic scale.

Materials and methods
A CBM approach for predicting metabolic biomarkers
Our method is applied to the genome-scale human metabolic network model of (Duarte et al., 2007). The model consists of 320 boundary metabolites that can be taken-up or secreted from human tissues through pseudo-reactions called exchange reactions. A positive flux through an exchange reaction represents the secretion of the boundary metabolite, while a negative flux represents its uptake. This model defines a space of feasible flux distributions that satisfy mass-balance constraints (embedded in the stoichiometric matrix S) and flux directionality constraints (embedded in the flux bound vectors \( v_{\text{min}}, v_{\text{max}} \)), as shown below in equations (1) and (2). Nonlinear thermodynamic constraints that are computationally harder to consider were not accounted for (Beard et al., 2002). We define the exchange interval for a boundary metabolite by determining the minimal and maximal value of its exchange reactions. The minimal and maximal values of an exchange reaction \( i \) are computed using FVA (Mahadevan and Schilling, 2003) by solving the following two linear programming optimization problems:

\[
\begin{align*}
\text{Min } v_i & \text{ or Max } v_i \\
\text{st} & \\
Sv & = 0 \\
v_{\text{min}} & \leq v \leq v_{\text{max}}
\end{align*}
\]

For each metabolic reaction \( r \) and every boundary metabolite \( m \) in the model, we compute the exchange interval of \( m \) when \( r \) is forced to be active (representing the healthy case), and when \( r \) is forced to be inactive (representing the disease case). To force \( r \) to be active in the healthy case, we constrain its flux to be larger than a flux activity threshold, denoted \( \varepsilon \), and compute the forward exchange interval \( H_r,m \). For reversible reactions, we also constrain the flux to be lower than \( -\varepsilon \), and compute the backward exchange interval \( H_r,m \). The healthy exchange interval \( H_r,m \) can be determined by taking the union of the forward and backward exchange intervals. However, as missing thermodynamic constraints in the model (which restrict reactions' directionality) may cause the backward exchange interval to falsely account for infeasible metabolic states, we tested a second method for computing \( H_r,m \), which considers the backward interval only in cases where the forward interval is predicted to be zero. Testing both methods for computing the healthy exchange interval showed a significant advantage to the second method while comparing our prediction with the clinical data obtained from Ramedis (yielding a precision that is 12% higher than that of the first method), and has hence been used in all further analysis. The activity threshold \( \varepsilon \) was set to a value of 1, and other thresholds in the range of 0.3–1 did not substantially change our results (less than 2% change in the predictions). To force reaction \( r \) to be inactive we simply constrain its flux to zero and compute the exchange interval \( D_r,m \). The commercial CPLEX solver was used for solving LP problems, on a Pentium-4 machine running Linux in dozens of milliseconds per each individual problem.

Metabolic biomarkers are predicted based on a comparison of exchange intervals between the healthy case and the disease case. For exchange intervals \( A = [a_1, a_2] \) and \( B = [b_1, b_2] \), we define:

\[ A < B \text{ if } (a_1 < b_1 \text{ and } a_2 \leq b_2) \text{ or } (a_1 \leq b_1 \text{ and } a_2 < b_2). \]

A metabolite \( m \) is predicted to be a biomarker of reaction \( r \) with an elevated extracellular concentration, if \( H_{r,m} \leq D_{r,m} \), and with a reduced extracellular concentration if \( D_{r,m} \leq H_{r,m} \). Biomarkers predicted with high confidence are determined similarly, but with the ‘\( \leq \)’ operator. To consider only significant changes between exchange intervals, a difference in flux, denoted \( a - b \), is considered only when \( a \) is at least 10% lower than \( b \). Selection of different sensitivity thresholds in the range of (5–15%) did not substantially alter our results (causing a change of less than 2% in our total predictions).

The prediction of biomarker alterations for gene knockouts that disrupt the activity of several reactions was performed by considering all biomarkers predicted for the affected reactions (based on the gene-to-reaction mapping in the model of Duarte et al and the list of disease causing genes extracted from OMIM given in Online Supplementary Dataset 1). In case of inconsistency between predicted elevated or reduced extracellular concentrations of a metabolite (once different reactions associated with the same gene are inactivated), we determine the biomarker state based on a majority rule, and consider it to be unchanged in case of a tie.

Validation using the RBC kinetic model
The RBC kinetic model consists of 43 metabolites, 43 internal reactions, and 12 primary exchange reactions (Jamshidi et al., 2001). The set of differential equations, describing metabolite concentration dynamics, were solved through Matlab’s ‘ode15s’ solver. Enzyme deficiencies were simulated by modifying the maximal rate constant of each enzyme in turn to 10% of its original value.

Automatic extraction of biomarker data from the OMIM database
A list of genetic metabolic diseases along with their causing genes was obtained from the OMIM database (McKusick, 2007). Biomarker data were extracted by parsing the disease description field in the OMIM database in search for metabolite names along with a mentioning of biofluids (e.g. plasma, urine). A dictionary of metabolite synonyms extracted from HMDB was used to resolve naming convention issues (Wishart et al., 2007).

Manual curation of biomarker data for amino acid-associated disorders
A set of in-born errors of amino-acid metabolism was obtained from the ICD-10 catalog of metabolic diseases (sections: E70–E72) as classified by the World Health Organization (World Health Organization, 2004). This set was reduced to include disorders that are cataloged in OMIM by their explicit ICD-10 name and are associated with a model gene (60 disorders). An additional 15 disorders that were predicted by our method to affect amino acids were added to this set. The known biomarkers of the amino acid-associated disorders compiled above were manually extracted from the disease description field in the OMIM database. The set of disorders was further filtered to include only the disorders that were reported to show a concentration change in at least one of the model’s boundary metabolites. This resulted in a final set of 17 disorders that composed the validation set (Supplementary Dataset 1).

Clinical measurements of biomarkers
The Rare Metabolic Disease database (Ramedis) hosts an extensive set of patients’ clinical data including measurements of metabolite concentration level in biofluids for 74 rare metabolic diseases (Töpel et al., 2006). Further data were obtained from HMDB, which records for each metabolite a list of normal and abnormal concentration levels in biofluids covering a set of 320 disorders (Wishart et al., 2007). Mining these databases for disorders that are associated with model genes and metabolites, as well as show a consistent view of metabolite concentration changes in both databases, resulted in a set of 29 metabolic disorders (Supplementary Dataset 1).
Supplementary information
Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

Conflict of interest
The authors declare that they have no conflict of interest.

Acknowledgements
We are grateful to Markus Herrgard for very valuable discussions and comments, from the inception of this study to its final form. MC is a fellow of the Edmond J Safra Program in Tel-Aviv University. TS is supported by an Eshkol Fellowship from the Israeli Ministry of Science. This research was supported by grants from the Israeli Science Foundation (ISF) to ER.

References
Akesson M, Forster J, Nielsen J (2004) Integration of gene expression data into genome-scale metabolic models. Metab Eng 6: 285–293
Beard DA, Liang SD, Qian H (2002) Energy balance for analysis of complex metabolic networks. Biophys J 83: 79–86
Cellarier E, Durano X, Vasson MP, Fargues MC, Demiden A, Maurizis JC, Madelmont JC, Chollet P (2003) Methionine dependency and cancer treatment. Cancer Treat Rev 29: 489–499
Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campo I, Czech M, Gross B, Hanspers K, Isserlin R, Kelley R, Killcoyne S, Lotia S, Maere S, Morris J, Ono K, Pavlovic V, Pico AR et al. (2007) Integration of biological networks and gene expression data using Cytopscape. Nat Protoc 2: 2366–2382
Covert MW, Knight EM, Reed JL, Herrgard MJ, Palsson BO (2004) Integrating high-throughput and computational data elucidates bacterial networks. Nature 429: 92–96
Duarte NC, Becker SA, Jamshidi N, Thiele I, Mo ML, Vo TD, Srinivas R, Palsson BO (2007) Global reconstruction of the human metabolic network based on genomic and bibliomic data. Proc Natl Acad Sci USA 104: 1777–1782
Durmus Tekir S, Cakir T, Ugen KO (2006) Analysis of enzymopathies in the human red blood cells by constraint-based stoichiometric modeling approaches. Comput Biol Chem 30: 327–338
Ito T, Tashiro K, Muta S, Ozawa R, Chiba T, Nishizawa M, Yamamoto K, Kuhara S, Sakaki Y (2000) Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc Natl Acad Sci USA 97: 1143–1147
Jamshidi N, Edwards JS, Fahland T, Church GM, Palsson BO (2001) Dynamic simulation of the human red blood cell metabolic network. Bioinformatics 17: 286–287
Kaddurah-Daouk R, Kristal BS, Weinshiboum RM (2008) Metabolomics: a global biochemical approach to drug response and disease. Annu Rev Pharmacol Toxicol 48: 653–683
Kenny LC, Dunn WB, Ellis DI, Myers J, Baker PN, Bell DB, GOPEC Consortium (2005) Novel biomarkers for pre-eclampsia detected using metabolomics and machine learning. Metabolomics 1: 227–234
Langher B, Brunetti-Pierri N, Lee B (2006) Inborn errors of metabolism: the flux from Mendelian to complex diseases. Nat Rev Genet 7: 449–460
Lee PJ, Briddon A (2007) A rationale for cystine supplementation in severe homocystinuria. J Inherit Metab Dis 30: 35–38
Levillain O, Balvay S, Peyrol S (2005) Mitochondrial expression of Arginase II in male and female rat inner medullary collecting ducts. J Histochem Cytochem 53: 533–541
Ma H, Sorokin A, Mazein A, Selkov A, Selkov E, Demin O, Goryanin I (2007) The Edinburgh human metabolic network reconstruction and its functional analysis. Mol Syst Biol 3:135
Mahadevan R, Schilling CH (2003) The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. Metab Eng 5: 264–276
McKusick VA (2007) Mendelian inheritance in man and its online version, OMIM. Am J Hum Genet 80: 588–609
Nicholson JK, Connelly J, Lindon JC, Holmes E (2002) Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 1: 153–161
Price ND, Reed JL, Palsson BO (2004) Genome-scale models of microbial cells: evaluating the consequences of constraints. Nat Rev Microbiol 2: 886–897
Reed JL, Patel TR, Chen KH, Joyce AR, Applebee MK, Herring CD, Bui OT, Knight EM, Fong SS, Palsson BO (2006) Systems approach to refining genome annotation. Proc Natl Acad Sci USA 103: 17480–17484
Seegmiller JE (1968) Detection of human inborn errors of metabolism by examination of urinary metabolites. Clin Chem 14: 412–417
Segre D, Vitiup D, Church GM (2002) Analysis of optimality in natural and perturbed metabolic networks. Proc Natl Acad Sci USA 99: 15112–15117
Shlomi T, Berkman O, Ruppin E (2005) Regulatory on/off minimization - metabolic flux changes after genetic perturbations. Proc Natl Acad Sci USA 102: 7695–7700
Shlomi T, Cabili MN, Herrgard MJ, Palsson BØ, Ruppin E (2008) Network-based prediction of human tissue-specific metabolism. Nat Biotechnol 26: 1003–1010
Shlomi T, Eisenberg Y, Sharan R, Ruppin E (2007) A genome-scale computational study of the interplay between transcriptional regulation and metabolism. Mol Syst Biol 3: 101–107
Stelling J, Klamt S, Bettenbrock K, Schuster S, Gilles E (2002) Metabolic network structure determines key aspects of functionality and regulation. Nature 420: 190–193
Tüpel T, Hofestädt R, Scheible D, Trefz F (2006) RAMESID: the rare metabolic diseases database. Appl Bioinformatics 5: 115–118
Urbanczyk-Wochniak E, Luemedemann A, Kopka J, Selbig J, Roessner-Tunali U, Willmitzer L, Fernie AR (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. EMBO Rep 4: 989–993
Vangala S, Tonelli A (2007) Biomarkers, metabolomics, and drug development: can inborn errors of metabolism help in understanding drug toxicity? AAPS J 9: E284–E297
Wagner M, Naik DN, Pothen A, Sakuriki S, Devineni RR, Adam B-L, Semmes OJ, Wright GL (2004) Computational protein biomarker prediction: a case study for prostate cancer. BMC Bioinformatics 5: 26
Wiback SJ, Palsson BO (2002) Extreme pathway analysis of the human red blood cell metabolism. Bioinformatics 18: 420–427
Xu K, Li S, Xiao W, Guo J, Zuo G, Jia Z, Sun C, Li X (2005) New method for the analysis of metabolite profiles of human tissue samples. Anal Biochem 341: 245–251
Xu G, Wang J, Yang J, Xu G, Liebich HM, Lutz K, Schmulling RM, Wahl HG (2003) Discrimination of type 2 diabetic patients from healthy controls by using metabolomics method based on their serum fatty acid profiles. J Chromatogr B 813: 53–58