Inborn errors of metabolism: the flux from Mendelian to complex diseases

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Abstract | Inborn errors of metabolism are characterized by dysregulation of the metabolic networks that underlie development and homeostasis, and constitute an important and expanding group of genetic disorders in humans. Diagnostic methods that are based on molecular genetic tools have a limited ability to correlate phenotypes with subtle changes in metabolic fluxes. We argue that the direct and dynamic measurement of metabolite flux will facilitate the integration of environmental, genetic and biochemical factors with phenotypic information. Ultimately, this integration will lead to new diagnostic and therapeutic approaches that are focused on the manipulation of these pathways.

Tandem mass spectrometry
Mass spectrometry (MS) is a technique that is used to identify compounds by their mass and charge. Compounds are typically separated, often by chromatography, and then ionized for detection. Tandem MS is a very useful high-throughput technology that uses two mass spectrometers in series: the first separates compounds, and the second identifies them based on their mass and charge.

Rhabdomyolysis
The breakdown of muscle tissue.

Cardiomyopathy
A disease process that alters the structure and function of the heart muscle; it is described as hypertrophic, dilated, or restrictive.

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doi:10.1038/nrg1880

Metabolism is the totality of all the chemical reactions that operate in a living organism. Inborn errors of metabolism (IEM) are genetic disorders that are caused by alterations of a specific chemical reaction in metabolism. For example, glycogen storage disease type I is caused by a defect in glucose-6-phosphatase, a crucial enzyme in glycogen breakdown. The resultant hypoglycaemia can be life-threatening; furthermore, the intermediate metabolites of glycogen breakdown are shunted to other pathways, which causes persistent hyperlipidaemia, hyperuricaemia and lactic acidosis[1].

Sir Archibald Garrod (1857–1936) coined the term 'inborn errors of metabolism' in 1908 (REF. 2) to describe genetically determined conditions — alkaptonuria, albinism, pentosuria and cystinuria — and is often regarded as the father of biochemical genetics (BOX 1). Strictly speaking, the term 'inborn' includes not only genetically determined disorders but also many conditions that are acquired by the foetus during pregnancy, such as intrauterine infections and exposure to teratogens. However, the use of 'inborn' has persisted, in part, in tribute to Garrod's foresight.

Although individually rare, IEM collectively account for a significant proportion of illnesses, particularly in children. In one Australian study, the prevalence of IEM as detected by newborn screening with tandem mass spectrometry was 15.7 per 100,000 births[3]. IEM can be pleiotropic, and can involve virtually any organ or system. For example, disorders of fatty-acid oxidation might present with hypoglycaemia, rhabdomyolysis, cardiomyopathy, hepatic disease or even maternal liver disease during the gestation of an affected foetus. Initial clinical presentation can occur any time from prenatal development through adulthood, and specific environmental triggers are crucial to determining an individual patient phenotype[4].

IEM have traditionally been regarded as Mendelian traits that are caused by single-gene mutations, and have served as important models for understanding mechanisms of monogenic disorders (BOX 1). However, as we learn more about the natural histories of IEM, we increasingly recognize that they represent the best examples of complex gene–environment interactions and, more specifically, gene–nutrient interactions that lead to complex disease[5,6]. IEMs are therefore a potentially powerful means to dissect both monogenic and common multifactorial diseases.

Many complex diseases can be perceived as specialized cases of IEM that originate from impairment of several steps in different biochemical pathways, each step being less severely affected compared to traditional Mendelian disorders (FIG. 1). Therefore, the net effect of a complex IEM on the organ and organism levels is the alteration of one or more metabolite fluxes. A metabolic flux is defined as the production or elimination of a quantity of metabolite per mass of organ or organism over a specific time frame (mole/kg/hr). In a classic IEM, one primary metabolite flux is affected. In complex disease, a network of metabolite fluxes might be subtly altered to cause a phenotype (FIG. 1). Ultimately, it is the concept of metabolic flux that is crucial in the translation of genotype and environmental factors into phenotype or a threshold for disease.

In this article, we explore an approach to IEM that illustrates the challenges that are presented by the study, diagnosis and treatment of these disorders. We predict

Box 1 | History of inborn errors of metabolism

Sir Archibald Garrod introduced the concept of inborn errors of metabolism (IEM) in an article that was published in *The Lancet* at the start of the twentieth century[2]. In the same article, Garrod proposed that inborn errors of metabolism were the emerging tip of a much larger iceberg of minor individual "errors" that affect different metabolic pathways. He defined this collection as constituting 'chemical individuality'; he understood that each person is biochemically unique and that individuality resulted from different enzymes that operated together in a way that was common to all individuals, but at the same time contributed to minor differences between individuals. Ironically, the work of Garrod was virtually ignored until the middle of the twentieth century, when extraordinary technological advances, including the development of electrophoresis and chromatography, facilitated a series of experiments that supported his theory on IEM[94]. Subsequently, Linus Pauling's work on sickle-cell anaemia demonstrated that genes could qualitatively alter protein structure and that mutations result in structurally different proteins[95], giving birth to the era of molecular medicine.

An important next step in the understanding of gene function and pathogenesis of genetic disorders was provided by Ingram, who showed that an alteration in a gene is indeed responsible for an amino-acid change in the corresponding protein sequence[96,97]. The link between genetic changes and protein alterations was soon extended beyond monogenic disorders. In 1969, Victor McKusick announced that "genetic factors are involved in all diseases"[98,99], thereby anticipating by over 30 years the concept that all human diseases have a genetic basis[100].

that the measurement and understanding of metabolic fluxes will enable us to overcome these challenges in both simple and complex IEM. We explore the classification, diagnosis, treatment and the underlying pathophysiology of IEM in general, with illustrative in-depth explorations of Gaucher disease (GD) and urea-cycle disorders (UCDs) as models for the evolution of the field. This discussion is especially timely, as expanded newborn screening via tandem mass spectrometry becomes more widely practiced in the US, Europe and other countries, and our ability to identify and manage both classical IEM and more complex metabolic derangements has greatly improved. We believe that progress in this field depends on moving away from the view that IEM are 'static' genetic and biochemical defects of simple pathways towards the concept of 'dynamic' metabolic fluxes, which reflect the metabolome.

IEM: classification, diagnosis and treatment

Classification. One of the primary challenges that is presented by IEMs is their extreme diversity, which has always made them difficult to classify. Many informal systems of classification have been proposed. IEM can be categorized according to the affected organ (as in 'neurological' or 'hepatic' diseases), the affected organelle (for example, 'mitochondrial', 'peroxisomal' or 'lysosomal' disorders) or the age of presentation (neonatal or adult-onset IEM). Because each of these approaches is informative, no single, universal classification system exists.

One of the more illuminating classification systems has been the separation of IEM into disorders that involve large or small molecules[7] (TABLE 1; Supplementary information S1 (table)). This classification system captures the wide spectrum of pathophysiology, clinical presentation and clinical management of inborn errors. Large-molecule diseases are generally associated with a gradual onset of disease; GD is an example, and is explored in a later section. By contrast, acute onset with remitting clinical course is often seen in small-molecule diseases, such as UCDs. There are obvious exceptions to these generalizations. For example, the 'large-molecule' glycogen-storage diseases can present with acute emergent hypoglycaemia.

The list of IEMs has expanded enormously in recent years, and so the list of diseases that is included in TABLE 1 and in Supplementary information S1 (table) can only be representative. The number will probably continue to grow until all variants of all enzymes and transporters that specify all homeostatic mechanisms in humans are identified.

Disease mechanism. The pathogenesis of an IEM can generally be attributed to the loss- or gain-of-function of mutant proteins (usually an enzyme or a transporter). The genetic basis of IEMs is extremely heterogeneous and can involve any type of genetic defect: one or more point mutations, deletions or insertions, or genomic rearrangements. Mutations can occur in coding or regulatory sequences, and mutations in different genes can phenocopy each other by affecting the same pathway. Disease is generally associated with altered metabolite flux through the pathway that is regulated by the mutant protein.

The biological effects of IEM mutations can be mediated by four main processes: direct toxicity of accumulating upstream metabolites; deficiency of downstream metabolites, that is, downstream substrate deficiency; feedback inhibition or activation by the metabolite on the same or different pathway; and diversion of metabolic flux to secondary pathways (FIG. 2). IEM might cause biochemical derangement, the effect of which might be systemic or localized to a specific organ or tissue. The effect can be cell autonomous or non-cell-autonomous.

All the above factors are crucial when considering potential diagnostic and treatment strategies. Examples of cell-autonomous functions include those that underlie classic enzymatic defects such as UCDs. To diagnose such a disorder, one might need to assay enzyme activity in an involved tissue. Because in this case the pathophysiology is intrinsic to each involved cell, effective therapy requires manipulation of the intracellular environment. Examples of non-cell-autonomous functions include the group of lysosomal enzymes that are secreted and then endocytosed distally via the mannose-6-phosphate receptor. In general, therapy for disorders with non-cell-autonomous pathophysiology might be directed at the organ or tissue where the damage takes place, rather than where the biochemical defect is located.

Disease diagnosis. Genomic and proteomic approaches to the primary clinical diagnosis of metabolic disease have been largely disappointing. Clear genotype–phenotype correlations have rarely been seen in IEM, so the primary method for disease evaluation and diagnosis has been through the analysis of metabolites. Unfortunately, the reliance on metabolites represents

Cell autonomous
Defective enzymes or proteins are said to act in a cell-autonomous fashion if their functions are confined within a cell.

Non-cell-autonomous
Enzymes and metabolites are said to act in a non-cell-autonomous fashion if they are produced in one cell but act in another cell, for example by affecting the transport of substrates between cells.

Genotype–phenotype correlations
Correlations between clinical severity and course with specific genetic variants.

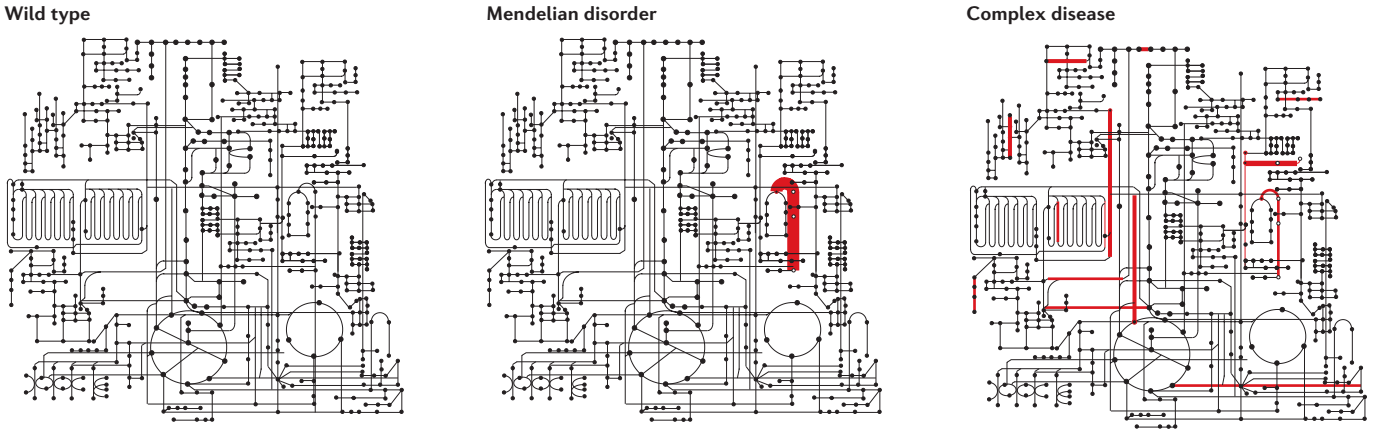

Figure 1 | **Inborn errors of metabolism and metabolic networks.** Affected pathways are shown in red and the degree of involvement is represented by proportional line thickness. In the case of a classic inborn error of metabolism, one primary metabolic pathway is affected. In the case of complex disease, numberous pathways are affected in a more subtle fashion.

a technological challenge. Genome, transcriptome and proteome profiling technologies each monitor molecules that have similar chemical properties. By contrast, the metabolome is comprised of chemically heterogeneous molecules that vary in different environmental states. Genomics and proteomics studies are generally carried out using microarrays and 2D gels or mass spectrometry, respectively, but the technique of choice is less obvious in the area of metabolomics, given the chemical heterogeneity.

There are several ways to diagnose an IEM, which depends on the specific disorder or presenting phenotype. Generally, measurements of metabolites in various body fluids are obtained: organic acids in urine, amino acids in the plasma and fatty-acid-conjugation products (acylcarnitines) are commonly used to screen for the accumulation of substrates that are upstream of a biochemical blockage or the deficiency of downstream products. Metabolic profiles that are obtained by these means, however, are necessarily incomplete, as each technique might only assay a specific class of compound. These tests might then lead to assays for more disease-specific enzyme activity, gene sequencing or other diagnostic procedures.

A broader, metabolomics approach can be obtained by using quantitative techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy (NMR spectroscopy)[8]. Recently, tandem mass spectrometry has facilitated the rapid and economical evaluation of a broad spectrum of metabolites from small samples, including dried newborn blood spots. This approach has enabled the timely diagnosis of many disorders, thereby facilitating early institution of therapy. Perhaps the best example has been the early identification of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency[9,10]. The broad application of tandem mass spectrometry to newborn screening will probably also reveal unanticipated intermediate biochemical phenotypes, as well as provide insights into their involvement in more complex phenotypes.

In addition to the quantitative assay of multiple metabolites, functional approaches are needed that incorporate dynamic measurements of the metabolome, ideally under steady-state conditions. A frequent approach to the diagnosis of IEM is to examine a profile of intermediary metabolites that have been extracted from a biological sample at a given time point. Ideally, if an enzymatic deficiency is present, metabolites upstream of the blockade will accumulate and there will be deficiency of those downstream. However, this approach, although useful and convenient in clinical care, is unrevealing when studying the higher levels of control and the interaction between metabolic networks. This strategy is further complicated by patients with partial, as opposed to null, enzymatic activity. In these patients, the biochemical phenotype might be normal until a specific physiological or catabolic stress causes decompensation.

The maturation in our understanding of metabolomics and metabolic flux has allowed a more comprehensive approach to the diagnosis, management and therapy of IEM, leading to improvements in the prognosis and outcome of certain disorders, as described later in the article.

Management and therapy. Given the extreme variability of IEM, their management and therapy has to be individualized for each patient, based on the patient's diagnosis and phenotype. However, the treatment of IEM broadly falls into a few categories. Certain IEM can be treated effectively with dietary intervention. A classical example is phenylketonuria (PKU), a disorder that is caused by phenylalanine hydroxylase deficiency. If phenylalanine is restricted from the diet, the toxic effects of phenylalanine accumulation are prevented and patients can develop with normal intelligence[11]. This discovery was central to the rationale for instituting newborn screening for metabolic disorders[12]. Dietary restriction of metabolites upstream of the block has become a central management strategy for many IEM. Alternatively,

Microarray
An array of DNA fragments of either genomic or cDNA sequences that are deposited on solid support and used to identify copy-number variation and level of gene expression.

Nuclear magnetic resonance spectroscopy
(NMR spectroscopy). An analytical chemistry technique that is used to study molecular structure and dynamics, and explores spectrum differences that are caused by the differential alignment of atomic spins in the presence of a strong magnetic field.

Intermediary metabolites
Compounds that are neither precursors nor endproducts of metabolic pathway. Their accumulation or deprivation in inborn errors of metabolism might have pathologic and diagnostic significance.

Phenylketonuria
(PKU). An autosomal-recessive inborn error of metabolism that leads to the deficiency of the enzyme that converts Phe to Tyr. Phe accumulation leads to mental retardation and other neurological problems. A low-Phe diet is an effective therapy.

dietary supplementation of deficient compounds that are downstream of the block might be useful in IEM. The use of corn starch might alleviate the hypoglycaemia that is faced by patients with glycogen storage diseases[13]. Supplemental calories and the avoidance of fasting and catabolism is crucial to avoid the metabolic crises that occur in disorders of fatty-acid oxidation[14] (TABLE 1; Supplementary information S1 (table)).

For certain IEMs, replacement of the deficient enzyme is possible. This has been remarkably effective for GD type I, and is now clinically available for other storage diseases, including Fabry disease and some of the mucopolysaccharidoses[15]. Other disorders are currently under investigation, including Pompe disease[16].

Another approach to treating the storage diseases has been substrate-reduction therapy. In type I GD, the

Table 1 | **Examples of large- and small-molecule Mendelian inborn errors of metabolism***

Molecule/pathway affected	Representative disease	OMIM #	Clinical presentation	Management options
Large-molecule diseases				
Complex lipid degradation	Gaucher disease Type I	230800	Hepatomegaly, splenomegaly, bony lesions, acid β-glucosidase deficiency.	Enzyme replacement; substrate reduction.
Mucopoly-saccharidoses	MPS I (Hurler syndrome)	607014	Corneal clouding, mental retardation, hernias, dysostosis multiplex, hepatosplenomegaly. Elevated urine excretion of glycosaminoglycans, α-L-iduronidase-activity deficient.	Bone marrow transplantation. Enzyme replacement.
Glycogen storage diseases	GSD1A (Von Gierke disease)	232200	Hepatomegaly, pancreatitis, hypoglycaemia, hyperlipidaemia, hyperuricaemia, lactic acidosis. Liver adenomas and hepatocellular carcinomas.	Avoid hypoglycaemia and acidosis with continuous feeding or supplementation with uncooked cornstarch. Liver transplantation for carcinoma or adenoma.
Peroxisomal diseases	Zellweger syndrome	214100	Dysmorphic features, hepatomegaly, polymicrogyria, severe mental retardation. Pipecolic acidaemia, elevated long-chain fatty acids. Death usually in first year.	Symptomatic.
Small-molecule diseases				
Amino-acid-metabolism disorders	Phenylketonuria	261600	Microcephaly, pale pigmentation, mental retardation (if untreated). Teratogenic effects on foetuses of affected mothers (if untreated).	Phenylalanine-restricted diet.
Urea-cycle disorders	Ornithine transcarbamylase deficiency	311250	Hyperammonaemia, vomiting, coma. Episodic hyperammonaemia. X-chromosome-linked inheritance.	Acute: detoxification with dialysis, nitrogen scavengers. Long-term: protein restriction, nitrogen supplementation, arginine supplementation, nitrogen scavengers. Liver transplantation.
Organic acid-metabolism disorders	Methylmalonic acidaemia	251000	Mental retardation, episodic acidosis, hyperammonaemia, stroke-like episodes, progressive renal failure, neutropenia, cardiomyopathy.	Dietary restriction of propiogenic amino acids; carnitine supplementation. Consider liver transplantation.
Monosaccharides	Galactosaemia	230400	Progressive symptoms after start of milk feeding. Vomiting, jaundice, liver failure, sepsis, cataracts.	Lactose free, galactose-restricted diet.
Pyruvic-acid and lactic-acid metabolism disorders	Pyruvate carboxylase deficiency	266150	Psychomotor retardation, lactic acidosis, hypotonia.	Carbohydrate restricted diet. Cofactor supplementation.
Fatty-acid metabolism	Medium-chain acyl-CoA dehydrogenase deficiency	201450	Episodic hypoketotic hypoglycaemia. Decompensation with fasting or illness.	Avoid fasting and catabolic stress. Carnitine supplementation.
Purine- and pyrimidine-metabolism disorders	Adenosine deaminase deficiency	102700	Severe combined immune deficiency (SCID).	Bone marrow transplant.
Cholesterol biosynthetic disorders	Smith-Lemli-Opitz syndrome	270400	Dysmorphic features, midline malformations, severe psychomotor retardation.	Cholesterol supplementation. Consider HMG-CoA reductase inhibitors.
Vitamin and cofactors-metabolism defects	Biotinidase deficiency	253260	Metabolic acidosis, neurologic impairment, seizures, skin and hair defects.	Biotin supplementation.
Heme synthesis	Acute intermittent porphyria	176000	Abdominal colic, episodic events mimicking acute abdomen, polyneuropathy.	Avoid triggers. Treat acute attacks with IV glucose and hemin preparations.
Copper- and iron-transport disorders	Wilson disease	277900	Chronic liver disease, cirrhosis, psychiatric disease. Neurological deterioration.	Avoid copper in diet. D-penicillamine. Consider liver transplantation.

*A more extensive version of this table is available online. HMG-CoA, high mobility group-coenzyme A.

Sphingolipidosis
A disorder of the formation or breakdown of sphingolipids, a class of lipid that is derived from sphingosine. The most common disorder of sphingolipid metabolism is Gaucher disease.

Hepatosplenomegaly
An enlargement of the liver and spleen.

substrate synthesis inhibitor miglustat (N-butyldeoxynojirimycin) decreases glycolipid biosynthesis, thereby reducing the substrate for the glucocerebrosidase that is deficient in GD. This inhibitor, and similar approaches, are being actively explored in other storage diseases as well[17]. Finally, stimulation of alternative pathways for disposal of the accumulation of potentially toxic metabolites upstream of the block might be an effective tool in chronic management; this approach is best exemplified by the use of alternative-pathway medications that remove upstream precursors via other metabolic routes in the treatment of UCDs[18].

Therapy for IEMs might also involve the replacement of the defective gene. Currently, this approach is clinically available in solid-organ[19] or bone-marrow transplants[15]. IEM have also been an attractive target for virus-mediated gene therapy, as they might respond well to organ-specific transduction, although successful clinical translation has eluded the field[18,20].

Metabolomics and the importance of flux

Central to the identification and therapy of IEM is the concept of metabolite flux. The flux through specific biochemical pathways represents the dynamic input and output of chemical processes, and allows for the assessment of pools of metabolites in different system states. This is far more revealing than the static measurement of intermediary metabolites at a single time point, as it also accounts for the dimension of time[21,22]. Conceptually, IEM represent the clinical manifestation of the disruption of normal metabolite flux. Importantly, net flux represents the integration of genotype, transcription, translation and environment. Adequate therapy for IEM must restore sufficient flux of metabolites downstream of the block and decrease the flux of metabolites upstream of a block. This can be accomplished directly by increasing flux through the block, decreasing precursors that feed the pathway or diverting metabolites to other pathways.

In practice, to measure the flux of a specific compound, a small amount of a tracer is delivered to patients or to their cells in culture. Ideally, this tracer should be chemically identical but experimentally distinguishable from the endogenous compound for which the activity is to be assessed. The use of isotope-labelled tracers, which are measured by mass spectrometry, has spurred the development of new techniques to measure metabolite flux[23] (FIG. 3). Although radioactive tracers have been used *in vivo* in animals and *in vitro* in cell systems for decades, the safety of stable tracers (isotopes) has facilitated studies in humans[24]. The use of stable tracers has facilitated the kinetic study of many metabolic networks by providing an assessment of the integration of various complex control mechanisms[25,26].

As an example, the study of urea-cycle dynamics has been greatly enhanced by using stable isotopes in flux analysis. One significant challenge in the diagnosis and management of patients with UCDs is that the patterns of metabolic derangement that is characteristic of null-activity defects is not consistently present in patients with milder defects, who might still have devastating crises and decompensations. This concept is generally applicable to other IEM: patients with milder defects might escape diagnosis, and therefore treatment, before their decompensation. The development of techniques to measure metabolite flux might allow better identification of these patients, thereby allowing for earlier therapy.

For the UCDs, measuring urea production at steady-state protein intakes can distinguish between patients with null-activity, symptomatic partial-activity and asymptomatic partial-activity. For example, the measurement of urea flux distinguished heterozygote female ornithine transcarbamylase (OTC) carriers from their unaffected siblings[27]. The measurement of metabolite flux has also expanded our understanding of the pathophysiology of UCDs. Using these same techniques, it was shown that nitrogen that was mobilized from peripheral stores, such as during catabolic stress, is processed by the urea cycle less efficiently than dietary protein that is absorbed from the gut. This phenomenon might explain the clinical observation that patients who have hyperammonaemia in the setting of an infection are often more difficult to manage than those whose decompensation is triggered by ingesting too much protein[28].

To illustrate the complexities of IEM and the various applications of metabolite-flux concepts to the diagnosis, treatment and ongoing research into these disorders, we explore two groups of conditions that illustrate both the achievements and challenges for the future. We have chosen GD and UCDs as model examples of large- and small-molecule disorders, respectively.

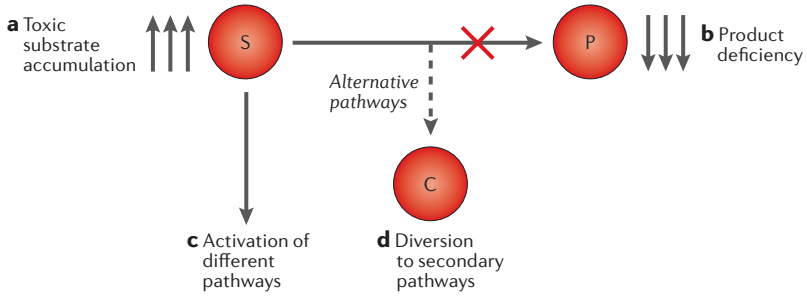

Figure 2 | **Pathogenetic mechanisms in the inborn errors of metabolism.** The effect of an enzymatic blockage results in: (**a**) direct toxicity of the accumulating upstream substrate (S); (**b**) deficiency of the downstream product (P); (**c**) activation of alternative pathways; (**d**) diversion of metabolic flux to secondary pathways and alternative metabolite (C) production. Large-molecule diseases often arise from the aberrant synthetic or degradative processing of polymeric molecules, for example, glycoproteins and glycolipids. Small-molecule diseases often involve small organic acids, amino acids, glucose, fatty acids, nucleotides and ammonia. In recent years, it has become clear that deficiencies of intermediary metabolites can also affect the overall activity of a specific biochemical pathway, and that this can be caused by the altered transport of metabolites within the cell and/or outside the cell. For example, primary carnitine deficiency, which is caused by a defect in a carnitine transporter that is expressed in the kidney, can lead to secondary inhibition of the urea cycle and hyperammonaemia[101].

Large-molecule disorder: Gaucher disease

Clinical manifestation. GD is a good example of a 'classic' IEM that is also a 'complex disease'. GD is a sphingolipidosis that is characterized by the impaired activity of the lysosomal enzyme glucocerebrosidase[29] owing to mutations in the glucocerebrosidase (*GBA*) gene.

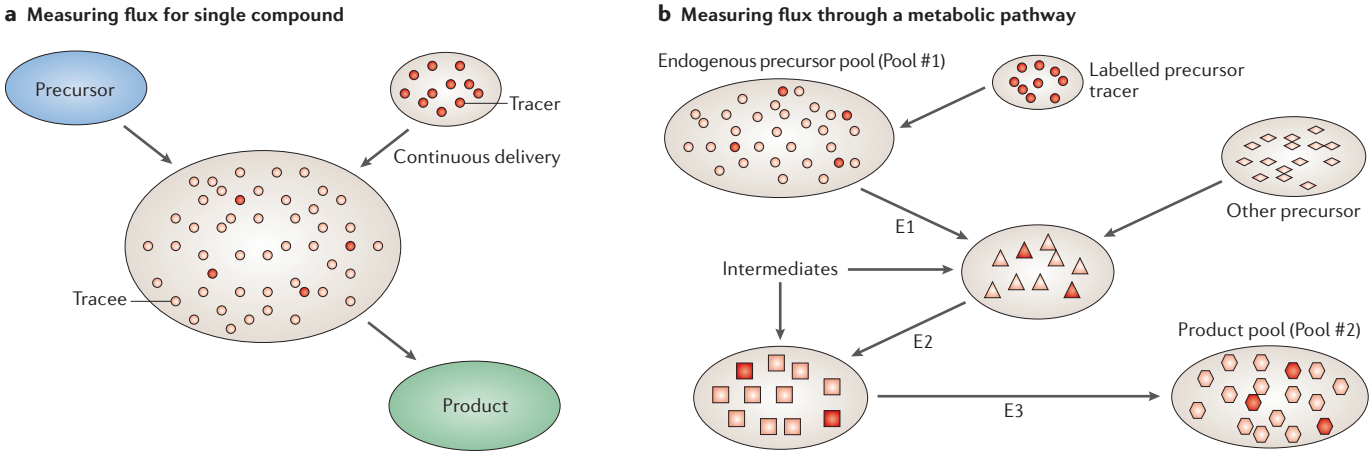

Figure 3 | **Measurement of metabolite flux using isotope tracers.** To measure metabolite flux using stable isotopes, the compound of interest must first be identified. The endogenous, naturally occurring compound is the 'tracee'. The labelled compound that is delivered to the subject experimentally is the 'tracer'. Typically, the tracer is chemically identical to the tracee, other than the presence of one or more atomic-isotope species that is incorporated into the tracer. For example, organic compounds might have a ^{13}-C incorporated, giving the tracer a molecular mass of one unit greater than the endogenous compound. The tracer is then given to the subject in very small quantities, so that the physiological processes being measured are not perturbed. Then, the isotopic enrichment of the tracee can be measured by mass spectrometry. The tracer disperses in the body into various 'pools'. These pools are physiological compartments that can be separately sampled. For example, a tracer might be split among intravascular, extravascular and intracellular pools. One can assess isotopic enrichment of the tracee in blood, urine, cerebral spinal fluid or any other compound of interest. These techniques can be applied to the flux of a single compound or of a metabolic pathway. **a** | Measurement of flux for a single compound. A tracer is introduced in a continuous fashion. As it accumulates in the tracee pool, it reaches a steady state. At steady state, the enrichment of the tracee by the tracer will provide the rate of appearance (flux) of the metabolite. Flux is measured as the (rate of infusion)/(isotopic enrichment of tracee). This measurement allows us to assess the dynamic input into the 'tracee' pool from precursors that contribute to its synthesis, as well as products that contribute to its elimination. **b** | Measuring metabolic flux across a pathway. Here, the label in the tracee pool can in fact be measured after it is transferred into the product pool. Labelled substrate is introduced into a precursor pool (Pool #1) *in vivo*. The substrate is then processed via a series of enzymatic steps (E1, E2, E3) and combined with other precursors until the final product is produced, the product pool (Pool #2). By measuring the percent enrichment of both Pool #1 and Pool #2 with the isotopic label, the fractional transfer of label from substrate (tracer) to product can be assessed. Fractional transfer from precursor to product is measured as the (isotopic enrichment of product)/(isotopic enrichment of precursor).

Clinical features of GD include hepatosplenomegaly, anaemia, thrombocytopenia and bone-marrow infiltration with characteristic storage cells and bony lesions[30]. The disease is generally progressive, with an unremitting course, and is the prototype 'large-molecule disease'. GD has classically been divided into three clinical phenotypes that are distinguished by the presence or absence of neurological manifestations.

Genotype–phenotype correlation. More than 200 different mutations in the *GBA* gene have been causally associated with GD, and this allelic heterogeneity might help to explain the clinical heterogeneity of the disease[31,32]. In contrast to the generally poor genotype–phenotype correlations in IEM, two GBA mutations, at amino acids N370S and L444P, have predictive value on clinical outcomes[32]. The presence of the N370S mutation on one single allele predicts non-neuronopathic disease, whereas homozygosity for N370S predicts a less severe disease phenotype in adults. However, there is still significant phenotypic heterogeneity among patients with the same genotype, implying that although mutations in the *GBA* gene are required to cause GD, other factors are important in the manifestations of the disease.

Molecular pathology. GBA is a lysosomal membrane-associated glycoprotein and, as such, is translated by ER-bound polyribosomes that are glycosylated through ER translocation, further modified while traversing the Golgi apparatus and finally transported to lysosomes. Each of these steps can be modified by polymorphism in other proteins, thereby further contributing to phenotypic heterogeneity. For example, variants that generate misfolded proteins might be eliminated by the ubiquitin–proteasome pathway[33–38]; the degree of decrease in protein level can contribute to disease severity in GD, and therefore to phenotypic variation. Clearly, the phenotype of each patient is the result of many genotypes that can compensate or aggravate a defect in *GBA*. At the extreme end of the spectrum, there are mutation-carrying octogenarian patients who do not present with any disease features[39]. GD therefore illustrates a continuum from a classic monogenic disease to a complex one.

Gaucher disease and flux. Measurement of metabolite flux is a key tool in the ongoing study of the pathophysiology of GD. For example, by measuring the incorporation of isotopically labelled methylcholine into phoshatidylcholine and sphingomyelin, Bodennec *et al.*

Thrombocytopoenia
A decrease in the number of platelets in the blood.

showed that the upregulation of phosphatidylcholine synthesis occurred in neuronal mouse models of GD[40,41]. This study[40] is one of many examples of the use of isotopic tracers to examine the activity of this pathway in various experimental models. Although the measurement of metabolite flux *in vivo* has not yet become practical in the routine clinical diagnosis and management of patients with GD, the alteration of flux is central to its management, as described below.

Management. The approach to therapy for GD has evolved markedly. Before specific treatment for GD was developed, only symptomatic therapy could be offered, as for other IEM. For many years, splenectomy was indicated to counteract life-threatening severe anaemia and thrombocytopenia. However, these and other early therapies for GD did not address the underlying biochemical defect.

Enzyme-replacement therapy became available in 1991 (REFS 42–44), and this is now the mainstay of the current management for GD; splenectomy is used only rarely. Enzyme replacement works by normalizing the flux of the degradation pathways of sphingolipids to prevent their accumulation and the subsequent tissue damage. In general, the response to enzyme replacement is excellent, regardless of disease severity[45,46]. Approximately 10–15% of patients develop antibodies against the enzyme, but these are almost always non-neutralizing[47].

More recently, substrate-reduction therapy has given promising results and will probably be a significant adjunct to enzyme replacement. Conceptually, tissue storage is reduced by limiting the substrate of the enzyme to a level that can be cleared by residual enzyme activity. This approach addresses the metabolite flux by decreasing the inflow of precursors. It is unknown whether the reduction of these substrates, which are important structural lipids, will have any unforeseen adverse consequences. One of these substrate reducers, miglustat, has proceeded through clinical trials[17,48]. Finally, gene transfer that is directed to haemopoietic cells is an area of active research for future therapies[49]. These continuing investigations are crucial, as there continues to be little effective therapy for GD types II and III.

The success with GD demonstrates how one can correct a disease phenotype by normalizing flux by increasing activity (enzyme replacement) or by decreasing precursors (substrate reduction). It also demonstrates how understanding the non-cell-autonomous distribution of the glucocerebrosidase enzyme led to the first success in protein-replacement therapy for IEM, something now replicated for storage diseases such as Fabry disease[50], as well as others[51,52].

Gaucher and complex disease. Although classical GD is a complex and challenging IEM, it also teaches us how more common 'complex' disorders can derive from the pathogenesis of 'simple' IEM. Heterozygosity for a non-neuropathic *GBA* mutation is a susceptibility marker for Parkinson disease (PD)[53,54]. At the neurological level, GD and PD share pathophysiological features, such as neuronal loss, astrogliosis, and the presence of intraneuronal lewy-body-like inclusions in the hippocampus[55]. However, only a few *GBA*-mutation carriers develop PD. This might be explained by the presence of efficient genetic mechanisms that either prevent the deposition and accumulation of glucocerebroside in dopaminergic neurons or that adequately degrade the glucocerebroside that is deposited. Understanding these mechanisms will shed light on the pathogenesis of other forms of PD and help to identify GD patients who are less prone to develop PD. As our understanding of this relationship increases, it will probably inform and deepen our understanding of both disorders.

GD is an excellent example of how a large-molecule disease results from the loss of a lysosomal degradative enzyme. Although the enzyme acts in a cell-autonomous fashion, it might be therapeutically delivered to other tissues via uptake with the mannose-6-phosphate receptor. Understanding this mechanism led to the first real success for enzyme-replacement therapy. Moreover, the pathogenesis of the disease exemplifies how the accumulation of the upstream metabolite leads to direct toxic effects on a cell-autonomous basis. Measuring net metabolic flux would be helpful in understanding why some carriers are at risk for PD and some are not; however, the application of tracer studies has been more readily achieved for small-molecule than for large-molecule diseases such as GD.

Small-molecule disease: urea-cycle disorders

Urea-cycle disorders (UCDs) provide a unique and revealing perspective on the history and future of IEM and the layers of complexity within the metabolome. The urea cycle[56] is the primary mechanism by which excess nitrogen that is generated by endogenous protein catabolism or exogenous protein intake is detoxified and excreted as urea[57]. Classic UCDs are caused by enzymatic deficiencies in this pathway, and their prevalence is at least 1/30,000, with partial deficiencies probably being more common. The most common UCD is ornithine transcarbamylase deficiency (OTCD) (FIG. 4).

Clinical presentation. The clinical presentation of UCDs can be heterogeneous. Classically, patients with null activity in proximal urea-cycle enzymes present in the neonatal period with severe hyperammonaemia, whereas patients with residual enzyme activity and residual urea-cycle flux present late into childhood or into adulthood with symptoms that range from cyclic vomiting to psychiatric disorders[58–61].

Molecular pathology. Loss of urea-cycle enzymes leads to the accumulation of ammonia upstream of the enzymatic block and deficiency of arginine downstream (except in the case of arginase deficiency; see FIG. 4). From a pathogenic perspective, ammonia acts in a non-cell-autonomous fashion, as its primary toxic effects are on the CNS. However, patients also have other clinical features such as primary liver dysfunction, which is most markedly seen with argininosuccinic acid lysase deficiency in argininosuccinic aciduria (FIG. 4).

Splenectomy
The surgical removal of the spleen.

Astrogliosis
An abnormal increase in the number of astrocytes, typically owing to neuronal cell death or injury.

Lewy body
An abnormal aggregate that is seen in nerve cells of patients with Parkinson disease or lewy-body dementia.

Hippocampus
An area of the brain within the temporal lobe. It is thought to have a crucial role in the limbic system.

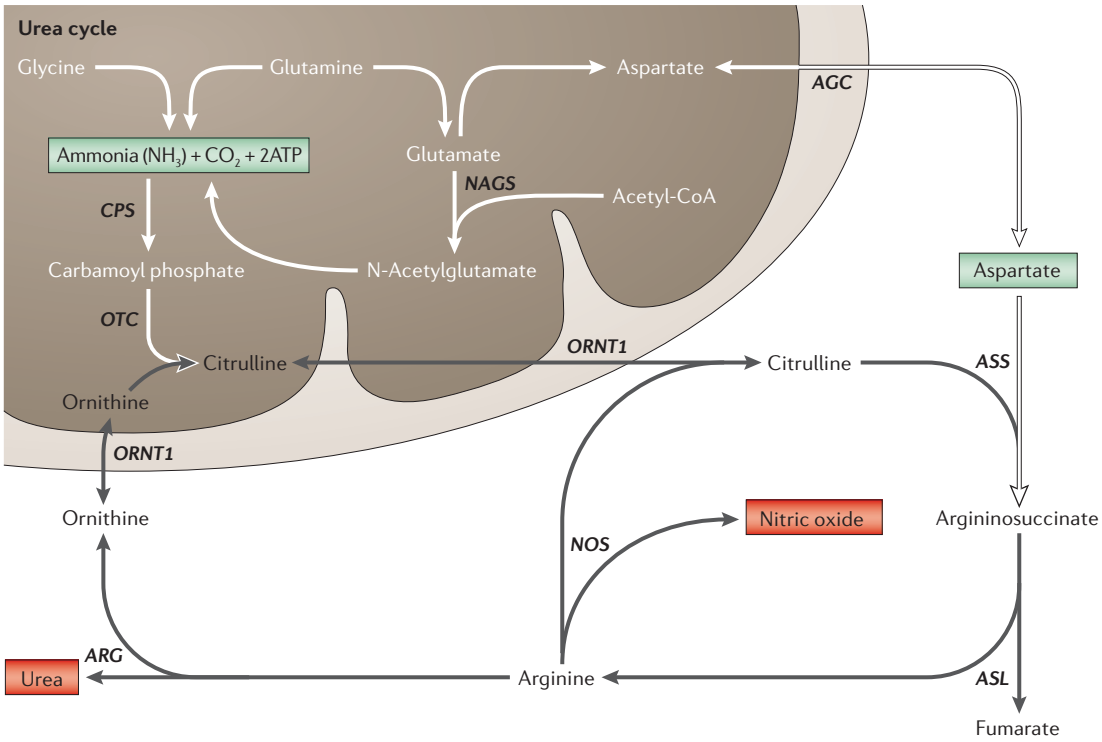

Figure 4 | **Urea-cycle disorders, small-molecule diseases.** The urea cycle consists of a group of enzymes that generate urea from nitrogen that is donated from ammonia and aspartate. Embedded within the urea cycle is the arginine–citrulline cycle, which produces nitric oxide. Disease-causing mutations have been identified in all enzymes and transporters shown here. The most common urea-cycle disorder is ornithine transcarbamylase deficiency (OTCD, OMIM 311250). The other urea-cycle disorders are deficiencies of N-acetyl glutamate synthetase (NAGS, OMIM 237310), carbamyl phosphate synthetase (CPS, OMIM 237300), argininosuccinic acid synthetase (ASS, OMIM 215700), argininosuccinic acid lyase (ASL, OMIM 207900, also known as argininosuccinic aciduria (ASA)), and arginase I (ARG1, OMIM 207800). AGC, aspartate-glutamate carrier (citrin); NOS, nitric-oxide synthase; ORNT1, ornithine translocase-1.

The phenotypic differences among the UCDs also highlight the importance of urea-cycle intermediates in other pathways of the metabolome[62–68]. For example, although arginine is a urea-cycle product, it is also the primary substrate for nitric oxide (NO) synthase (FIG. 4). Therefore, the clinical features of the UCDs that are not related to hyperammonaemia might in part be due to dysregulation of NO synthesis (see REFS 69,70 for evidence that indicates that metabolite channeling occurs).

The compartmentalization of urea-cycle enzymes in different organs requires the inter-organ flux of intermediary metabolites; the same principle applies to the intracellular compartmentalization of urea-cycle intermediary metabolites. Mitochondrial and plasma-membrane transporters facilitate the transport of intermediary metabolites (such as aspartate and arginine) that are required for normal urea-cycle flux. Loss of these transporters leads to local depletion of urea-cycle intermediates, which leads to decreased urea-cycle activity despite normal urea-cycle enzyme function[18].

Diagnosis. The clinical approach to the diagnosis of UCDs illustrates the evolution of our understanding of IEM. Initially, the diagnosis is indicated by elevated ammonia in the absence of significant metabolic acidosis[71]. After the quantification of plasma amino acids became more readily available, specific enzymatic defects were extrapolated from alterations of a basic metabolic profile — that is, increased upstream and decreased downstream metabolites. This snapshot metabolomic approach could reliably identify null-activity patients, but it has been unreliable in identifying partial-activity patients or carriers because non-steady-state metabolite pools are often normal in the absence of stress. The definitive diagnosis of enzyme deficiencies became feasible when direct enzyme assays on fibroblasts or hepatocytes became clinically available[57]. However, because of technical limitations, such as the presence of substrate excess *in vitro*, enzyme activities do not necessarily correlate with phenotypic severities. Finally, the molecular identification and sequencing of urea-cycle genes have facilitated specific diagnoses, although their sensitivity is not 100%[72].

One of the more vexing challenges with UCDs (and IEM in general) has been establishing a reliable approach to identify partial-activity patients, such as female carriers of X-chromosome-linked OTCD or male patients with mild deficiencies. This is a common problem in IEM, in which subtle alterations of enzymatic activity might only cause clinical symptoms when the respective pathway is stressed by increased flux. The phenotype of

females with OTCD ranges from severe hyperammonaemia in the newborn period to a completely asymptomatic state, because of skewed hepatic X-chromosome inactivation[73,74]. Provocative tests that 'stress' the target pathway, such as protein challenge, have been used to diagnose partial-deficiency states. However, these tests can be associated with significant variability and potential morbidity. An alternative diagnostic tool is based on stimulating metabolite production through a secondary pathway (see REFS 73,75 for examples). However, this method might also be confounded by false positives, owing to patients with deficiencies of the respiratory chain, which further underscores the interconnection of the metabolic network.

Direct *in vivo* measure of ureagenesis potentially provides the most accurate measure of total urea-cycle activity — that is, of metabolic flux. It represents an approach to understand the consequence of subtle genetic changes on flux through a complex metabolic network. Multiple stable-isotope tracer assays have been explored[27,76]. For example, primed-constant infusion of glutamine that is labelled with ^{15}N in the amide group or labelled ammonium can be used as a nitrogen donor in a precursor pool. This stable isotope will be transferred into the urea cycle via carbamyl phosphate synthesis. The enrichment of this label in precursor and product pools, which is measured by mass-spectroscopic methods, can be used to assess the fractional transfer of nitrogen from the glutamine or ammonia into urea, which provides a reliable measure of *in vivo* ureagenesis or flux. These methods can distinguish neonatal-presenting patients, late-onset patients, asymptomatic carriers and unaffected individuals. In fact, the ratio of labelled urea and glutamine directly correlates with clinical severity[27].

Therefore, the measurement of metabolic flux under steady-state conditions provides a dynamic assessment of multiple metabolic pathways, which are integrated with environmental factors such as diet, to represent phenotypic severity[77]. However, although physiological measures of metabolite flux might represent the most relevant assessment of the metabolome in the whole organism, technological and practical burdens, particularly cost and time requirements for the assays, have hampered their widespread application to routine clinical use.

Management. The uniformly poor outcome of neonatal presenting cases has improved with the development of alternative-pathway therapy via the use of sodium phenylacetate, sodium benzoate and, more recently, sodium phenylbutyrate[78]. These medications bind glycine and glutamine, which allows these compounds to be excreted through the kidneys. As these are key ammonia donors to the urea cycle, this treatment diverts the flux of nitrogen from the urea cycle into these alternative pathways. The development of alternative-pathway therapy is an important paradigm in the treatment of IEM — that is, diverting metabolite flux away from the affected pathway[79]. A complementary approach is to limit exogenous and endogenous sources of the offending nutrient, which, for UCDs, include nitrogen-containing compounds. Limiting exogenous sources can be achieved by restricting protein intake, whereas endogenous sources can be limited by preventing catabolic stress that stimulates the mobilization of amino acids from muscle.

UCDs and complex disease. Similar to the GD example, subtle dysregulation of the urea-cycle flux might contribute to complex diseases. In this case, the best-characterized example is the association between the T1405N polymorphism in the carbamyl phosphate synthetase-1 (*CPS1*) gene and plasma levels of arginine/citrulline, with the risk of persistent pulmonary hypertension in newborns[80]. The same polymorphism has also been implicated as a risk factor for veno-occlusive disease after bone marrow transplantation[81–83]. Interestingly, these studies support the hypothesis that altered flux through the urea cycle can secondarily affect the nitric-oxide metabolism in the pathogenesis of distinct, complex phenotypes[84].

The need for an integrated approach

Despite the considerable effort and expense that have been expended on whole-genome screens that aim to detect genetic loci that contribute to the susceptibility to complex human diseases, the results have often been inconsistent[85,86]. With few specific exceptions, such as GD, the search for genotype–phenotype correlations has largely been disappointing. It is possible that an individual genomic variant affects metabolite flux in a subtle fashion, without evidence of clinical disease. The cumulative effects of multiple sequence variations on a network of metabolite fluxes might, however, be sufficient to surpass the clinical threshold for disease, either alone or in combination with environmental factors[87].

Metabolism is therefore far more complicated and intertwined than can be accounted for simply by the 'one gene–one enzyme' model, and so the genome alone might turn out to be insufficient to explain the complexity of human metabolic diseases[88]. Variation in biological systems indicates the potential for pathophysiological effects that are attributable to feedback mechanisms and secondary alterations in metabolic flux that might not be completely reflected in gene polymorphisms and gene-expression changes[89].

So, to understand the effects of genomic changes on the biological system, knowing the genome is not enough — we need to know the relative concentrations of the RNA molecules, that is, the transcriptome; the relative concentration of the proteins, that is, the proteome; the nature of protein–protein interactions, that is, the interactome; as well as the concentrations of the metabolites that exist in any metabolic and environmental state, that is, the metabolome (FIG. 5).

Integrative approaches for sampling the biological organism while simultaneously taking into account all of these factors is the best hope for understanding the complexities of IEM and human variation in disease[90]. The dynamic measurement of metabolite flux through

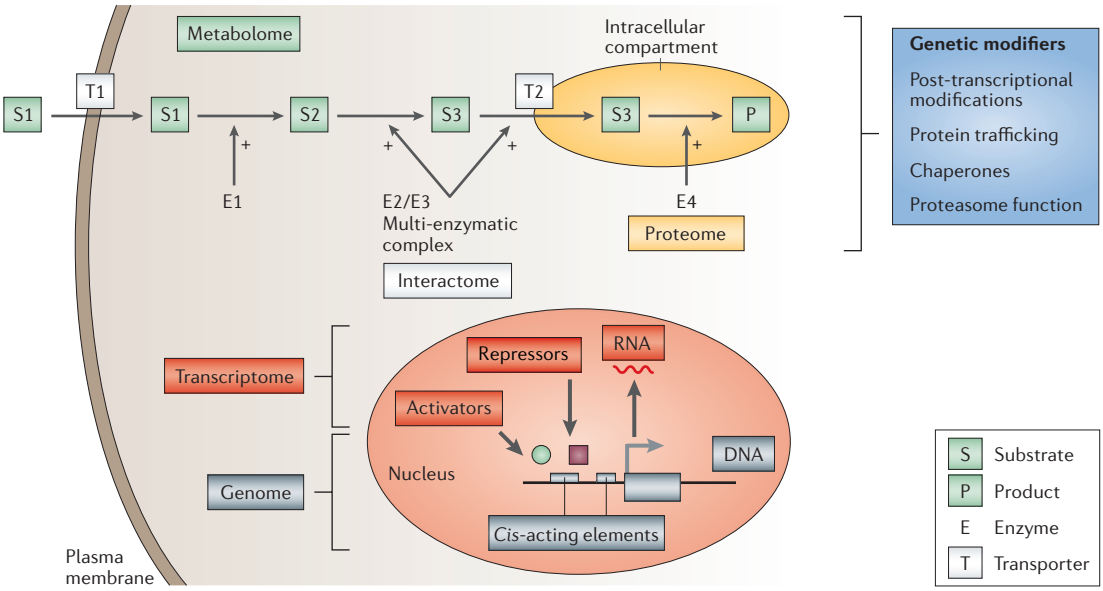

Figure 5 | **Metabolic pathway and layers of phenotypic complexity.** The complex interrelationship between the genome, transcriptome (the relative concentrations of RNA molecules) proteome (the relative concentration of proteins), interactome (the nature of protein–protein interactions) and metabolome (the concentrations of the metabolites that exist in any metabolic and environmental state). Alterations in any of these levels of control might influence the final phenotype.

the body and the organ might offer one of the best windows into this complexity. We argue that integration can be achieved by associating the genotype not only with clinical and biochemical phenotype, but measurements of metabolic flux in 'steady-state' conditions that control for environment–gene interactions.

To broaden our understanding of genotype–phenotype relationships, we must apply metabolite-flux measurement to other areas of metabolism. Until now, flux measurements have lent themselves to relatively 'isolated pathways', with more restricted inputs and outputs. The urea cycle is one such example. However, more complex pathways, such as that seen in the energy transfer through the respiratory chain, would be important targets, given the widespread clinical consequences of energy-deficiency states.

We anticipate that with further advances in the understanding of the metabolome and of the concept of metabolic flux, IEM will continue to provide new and important breakthroughs in both classical genetic disorders and common complex disease.

Future directions

The study of the aetiology, pathogenesis and natural history of IEM has taken on greater importance outside scientific circles because of the expansion of comprehensive newborn screening using tandem mass spectrometry. This screening approach will facilitate the early, often presymptomatic, detection of IEM by identifying abnormal metabolite levels in non-steady-state situations. However, although most defects identified will be *bona fide* classical Mendelian IEM, a portion of them probably labelled as false positives and with uncertain clinical significance might turn out to represent hypomorphic variants that affect metabolite flux in a more subtle fashion compared with classical IEM. Furthermore, they might be associated with complex, adult-onset phenotypes that have yet to be determined. Progress in genetic medicine will require us to understand these emerging natural histories of IEM, to apply molecular and biochemical methods for their diagnoses and to develop tools for evaluating the metabolome as a dynamic system that integrates multiple levels of genetic, biochemical and environmental controls. Such strategies have now been applied in IEM, such as galactosaemia, glycogen-storage disease and maple syrup urine disease, in addition to the UCDs, and more pleiotropic phenotypes, such as mitochondrial disease, await in the wing[91–93].

To advance our understanding of IEM from a metabolic-flux perspective, we will need technological and theoretical advances that include: the measure of metabolite flux for complex molecules, including whole proteins, polysaccharides and complex lipids; new modelling and experimental approaches to account for compartmentalization of metabolites within the cell and outside the cell between organs; and rapidly expanding the measurement of metabolite flux to the clinical arena. Although there have been successes, for example the development of a multiple-isotope-based breath test for the diagnosis of infectious and liver diseases, clinical applications for IEM are lacking. Ultimately, an integrated assessment of the individual metabolome will allow us to diagnose complex phenotypes, and the understanding of the pathogenetic mechanisms of IEM can help us to devise treatment strategies that manipulate metabolite flux in the disease states.

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Competing interests statement
The authors declare no competing financial interests.

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