Isovaleric acidemia: an integrated approach toward predictive laboratory medicine

Dercksen, M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 8

General discussion and future prospects
1. Context

Investigations of Mendelian disorders caused by defects in single genes, such as isovaleric acidemia (IVA), have progressively revealed the existence of non-specific genetic and phenotypic variations. These findings paved the way for genome-wide association studies (McCarthy et al., 2008), resulting in the assessment of underlying health risks and the disclosure of global metabolite profiles (Wikoff et al., 2007) for both rare and common diseases. The comprehensive investigations of genes, proteins and metabolic pathways in diseased and healthy individuals through powerful new applications, such as the "-omics" technologies, may well contribute to the development of optimized therapeutic strategies focusing on the individual, commonly known as personalized laboratory medicine. The success of personalized medicine depends not only on having accurate diagnostic tests consisting of state-of-the-art laboratory practices, but ideally also requires communication between physicians/paediatricians, clinical chemists/biochemists and geneticists/molecular biologists on their combined findings, interpretation and knowledge, which may become paramount for innovative approaches towards clinical and laboratory practice (Hall et al., 2004; Hamburg and Collins, 2010).

Against this brief background on contemporary tendencies with regard to IEMs, a primary goal of this thesis was to follow such an integrated approach by using a monogenetic disorder, IVA, as a model to investigate. The extensive knowledge of IVA (Vockley et al., 2012), the first organic acidemia to be described in literature (Tanaka et al., 1966), makes it an ideal model for the evaluation of an IEM through an integrated approach. The evolving characterization of IVA patients has steadily revealed the genetic and phenotypic heterogeneity of this disease, which further motivated the need for a personalized understanding of this disease (Vockley and Ensenhauer, 2006). The availability of biological material from a small IVA cohort in South Africa, with limited information about the clinical and biochemical features of these patients as well as no genotypical characterization of the IVD gene, provided a unique opportunity to understand the underlying genetics in relation to the disease. The intention of this study was that such understanding will ultimately lead to a laboratory protocol for the early identification and subsequent individualized treatment of conditions such as IVA. A general review of these topics, with a focus on IVA, is presented in Chapter 1, which includes the outline and aims of this study. What follows in this chapter is the emphasis on the link between the observations from the integrated approach to monogenic disorders (subsection 2) leading to perspectives on predictive laboratory medicine (subsection 3).
2. The integrated approach

The integrated approach adopted in this study consisted first of standard diagnostic protocols, which included clinical, biochemical and genetic characterization of the individual patients which formed part of the South African IVA cohort (Chapter 2). The biochemical study was subsequently expanded to include a metabolomics approach (Chapter 4) inspired by the high expectations among the clinical fraternity on the potential contributions of metabolomics towards predictive laboratory medicine (McCabe, 2010; Mamas et al., 2011) and personalized health in paediatric care (Baraldi et al., 2009). The outcomes of Chapters 2 and 4 prompted the examination of an aberrant pathophysiological event – hyperammonemia – as well as the nutritional status of treated IVA patients (Chapters 5 and 7) respectively. This study also demanded new methodologies, which are described in Chapters 2 and 6 (enzymological applications) as well as in Chapter 3 (a bioinformatics improvement).

The first aim of this study (refer to Chapter 1) dealt with the biochemical and molecular analyses on biological material of South African IVA patients, the outcomes of which are presented in Chapter 2. Most importantly, the investigation also revealed a conspicuous genetic and biochemical homogeneity within the cohort. First, all patients turned out to be homozygous for the c.367G > A mutation, which is responsible for the substitution of glycine at position 123 by arginine (Chapter 2, Fig. 1). The genetic aberration in the IVD gene from the patient group was the first of its kind to be described for IVA (Dercksen et al., 2012). Studies on many IVA cases in populations around the world have revealed a wide array of mutations in the IVD gene, but no other example of the unique homogeneity of the IVA variant seen in the South African patients has been reported to date. Comparative results in this regard are presented in Table 1, which summarizes the IVD mutational studies reported in family groups from particular populations and further shows that these findings are unprecedented.

The observed genetic homogeneity of the South African IVA cohort strongly suggests a founder effect. It remains to be established, however, whether the 10 different IVA patients with the same homozygous mutation, but coming from 7 different families, can be traced back to the same ancestor as would be expected for a founder mutation. The use of bio-genealogy has been successfully applied in the laboratory for inborn errors of metabolism in Potchefstroom, South Africa, confirming a founder effect of osteogenesis imperfecta in South African cases (De Vries an De Wet, 1986; Knoll et al., 1988) and may well allow the determination of the inheritance pattern of this specific IVA cohort. Eventually, haplotype studies as well as population-genetic testing, which include determining the frequency of the mutation in the specific
Table 1: Summary of IVD gene mutations reported in various populations compared to the South African cohort.

<table>
<thead>
<tr>
<th>Countries</th>
<th>Number of patients</th>
<th>Gene/protein abnormalities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germany/US</td>
<td>12 German, 7 US patients (family relations not specified)</td>
<td>5 mild homozygotes (p.A314V) 8 mild heterozygotes (p.A314V) 6 heterozygotes (without p.A313V)</td>
<td>Ensenauer et al., 2004</td>
</tr>
<tr>
<td>Taiwan</td>
<td>6 (5 families)</td>
<td>1 homozygote and 5 different heterozygotes</td>
<td>Lin et al., 2007</td>
</tr>
<tr>
<td>Korea</td>
<td>8 (6 families)</td>
<td>2 (x2) different homozygotes and 4 different heterozygotes</td>
<td>Lee et al., 2007; Cho et al., 2013 Qiu et al., 2008; Lee et al., 2010; Bei et al., 2013 Vatanavicharn et al., 2011</td>
</tr>
<tr>
<td>China</td>
<td>3 (3 families)</td>
<td>3 different heterozygotes</td>
<td>Qiu et al., 2008; Bei et al., 2013 Vatanavicharn et al., 2011</td>
</tr>
<tr>
<td>Thailand</td>
<td>5 (5 families)</td>
<td>4 different heterozygotes and 1 homozygote</td>
<td>Hertecant et al., 2012</td>
</tr>
<tr>
<td>United Arab Emirates</td>
<td>6 (3 families)</td>
<td>3 different homozygotes</td>
<td>Hertecant et al., 2012</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>3 (1 family)</td>
<td>1 common homozygote</td>
<td>Kaya et al., 2013</td>
</tr>
<tr>
<td>South Africa</td>
<td>10 (7 families)</td>
<td>1 common homozygote (p.G123R)</td>
<td>Dercksen et al., 2012</td>
</tr>
</tbody>
</table>

* Note, only populations, with known mutations as well as more than one patient in an affected family, is presented in this table

Furthermore, homogeneity on a biochemical level was established by means of immunoblot analyses in fibroblasts from the IVA cohort, indicating the absence of the IVD protein (Chapter 2, Fig. 2). Moreover, the activity of IVD in fibroblasts from the patients was confirmed to be deficient (Chapter 2, Table 3). The activity of the IVD protein was not restored through the use of improved protein folding techniques (Chapter 2, Table 3). Therefore a treatment option with potential chaperones (Bernier et al., 2004) for improved folding of the tertiary structure of IVD will probably not be of any benefit for this IVA cohort.

In addition, it was thought to be desirable to provide further insight with regards to results presented in Chapter 2 Fig. 2, by using computational investigative tools to predict stability and consequently pathogenicity of a mutant protein. The use of in silico analyses to predict the stability of mutant IVD proteins has been informative in recent IVA case reports (Hertecant et al., 2012; Kaya et al., 2013; Bei et al., 2013). Consequently, an in silico analysis was conducted on the p.G123R IVD mutant with the computational application, PolyPhen-2 (Adzhubei et al., 2010). Fig. 1 illustrates the high degree of destabilisation within the corresponding p.G123R IVD protein. In
conclusion, the *in silico* analyse with PolyPhen-2 supports the observation of faulty structural formation of the IVD protein.

![Table and Diagram]

**Fig.1:** An illustration of the use of an *in silico* application i.e. PolyPhen-2, to predict the effect of mutation on the IVD protein structure. The HumVar result (specifically applied to Mendelian diseases) gives a score of 0.989 (indicated by the black line in the coloured spectrum), which suggests that the mutation has a severely damaging effect on the structure of the IVD protein. The high specificity of 0.94 supports pathogenicity of this mutation. The somewhat low sensitivity of 0.54 indicates that the probability of the correct classification may be affected due to different amino acid alignment criteria (Flanagan et al., 2010).

In contrast to the irrefutable genetic and protein homogeneity, the IVA cohort studied showed wide variability in terms of clinical symptoms (Chapter 2, Table 1) as well as varied metabolite levels associated with individual IVA patients (Chapter 2, Table 3). These findings were attributed to delayed diagnosis, the limited accessibility to NBS in South Africa, inconsistent treatment regimens and in some cases inadequate clinical follow-up sessions to assess the progression of the disease. In addition, a study of a larger cohort may disclose reasons for phenotypical as well as individual metabolite variation. Furthermore, other contributing factors, which were not investigated in this thesis, may complicate the clinical presentation of IVA. Such factors, which may inform on IVA heterogeneity, include acute or chronic bacterial or viral infections, environmental influences on additional modifier genes (epistasis), non-allelic mutations (polygenetic traits) and epigenetics (Budd et al., 1967; Dipple and McCabe 2000; Fletcher et al., 2012). Nevertheless, the protocol which was followed in this study provided evidence to successfully characterize the molecular and biochemical features of this specific IVA group and thereby satisfied the first aim set for this thesis.
The informative value of metabolomics prompted the metabolomic investigation of IVA, which became the second aim of this thesis (Chapter 1). The outcomes of this study are presented in Chapters 3 and 4. Three aspects of the metabolomics approach are highlighted in this discussion chapter: first, methodological aspects not covered in Chapter 3; second the relationship between the outcomes in the context of systems biology; and third, new biochemical insights that emanated from the metabolomics data obtained for untreated and treated patients.

- **The methodological aspect**

A semi-targeted approach (with focus on the organic acid components of the metabolome) was followed in three experimental groups affected by IVA – untreated, treated patients and adult carriers of IVA (obligate heterozygotes) – and compared to appropriate control groups (infants and children as well as adults, respectively) (Fig 2A). A condensed form of the four main components of the pipeline, which was followed in Chapters 3 and 4, is illustrated in Fig. 2 of this chapter for discussion purposes.

**A. Clinical description & case selection**
- 10 IVA cases = H0
- 10 IVA treated = HoT
- 12 Heterozygotes = Hh
- 54 Controls : 22 Infants = W
  32 Adults = A

**B. Metabolome selection & analysis**
- IVA = Organic aciduria
- Measurement design
- SOP for extraction and derivatization
- Blanks and sample distribution
- GC-MS analyses

**C. Identification and quantification**
- AMDIS: Peak deconvolution
- Identification: Custom made library

**D. Original normalized data**

**E. Data processing and reduction**
- Bioinformatics approach
- Biological filter / Manual curation

**F. Reduced normalized data**

Fig 2: A simplified representation of the experimental design followed in the IVA metabolomics study. The four main components of the pipeline are defined and illustrated in panels A, B, C and E. Drop-line representations of the original data and of the remaining metabolites after data reduction are shown in the figures linked to panels D and F, respectively. Metabolites indicated by I (N-isovalerylglycine) and II (a carbohydrate metabolite due to dietary treatment of the IVA cases) in the two drop-line figures are highlighted to illustrate the characteristic dynamic range of the metabolomics data, as well as the biological importance of metabolites with very different concentrations, as discussed in the text.
The analytical aspects of this study generated unique challenges:

First, approaches had to be devised to compare five different groups (See Fig. 2A). Dr. G. Koekemoer, expert on bioinformatics and part of the IVA multidisciplinary team, suggested the development of a new method to compare the global metabolite profiles of these groups, resulting in the development of the "concurrent class analysis" (CONCA), presented in **Chapter 3**. CONCA is a novel approach that is similar to a traditional principal component analysis (PCA) model with the added benefit of providing further information concerning each individual group and an ability to identify which variables have discriminatory power and are responsible for group separation. This benefit is clearly illustrated by the results from three groups (untreated IVA patients, treated IVA patients and children controls), which were used to evaluate the CONCA method, as shown in Chapter 3 (Table 1), and were successfully applied in the comparison of the five different case groups, which included the original 3 groups as well as the obligate heterozygotes and adult controls, in Chapter 4 (Fig. 2).

Second, the metabolite profiles of IVA were found to exhibit an extensive dynamic range (illustrated in Figs. 2D and 2F), dominated by N-isovalerylglycine (e.g. exceeding 1 000 mmol/mol creatinine, and at position I in Fig. 2D and 2F). The protocol designed for the GC-MS measurement of samples (Fig. 2B) can be summarized as: B-B-B-[S₁-B]…[Sₙ-B]. S₁ to Sₙ were randomly selected samples from all five experimental groups, in each case separated by a blank (B) (hexane, the carrier solvent). This measurement design was chosen to exclude the carryover effect of subsequent samples separated on the GC column.

Third, the samples analysed presented with many features at a very low level, which may include noise or potentially important indicators (e.g. drop-lines shown at position II in Fig. 2). In addition, metabolites derived from medication were also identified in some samples of patients as well as controls. Taken together, this resulted in an extensive number of features (more than 200) shown in the original data set (drop-lines below panel D in Fig. 2). The combination of zero reduction - a bioinformatics approach, as used in Reinecke et al. (2012) - and filtering of exogenous substances by manual curation - a biological approach, described in

---

1 Features and noise: In metabolomics, features indicate uncharacterized or unclassified chemical substances (as shown in Figure 2D and 2F above) or even a peak in a mass-spectrum, having a signal to noise ratio of 10 to 1. Noise will be those areas in raw metabolomics spectra of very low signal points. While the goal of the global metabolomics approach is to identify as many as possible features in a biological sample, the targeted approach differs in that the analytical method is designed to highlight metabolites of a particular class in an unbiased fashion (Griffiths et al., 2007).
Williams et al. (2012) - was used to produce reduced \(^2\) normalized \(^3\) data (drop-lines below panel E in Fig. 2). A \([n \times p]\)-matrix could subsequently be constructed from the reduced data, consisting of 86 cases (n) and 52 metabolites (p). This matrix was well suited for multivariate (PCA and PLS-DA) and univariate (effect size and Mann-Whitney tests) analysis. The bioinformatics analysis identified the important biomarkers for IVA (Chapter 4, Table 1) and identified metabolites associated with various treatment regimens (Chapter 4, Table 3).

The above overview of the metabolomics approach was included in this chapter to illustrate how it contrasts with the traditional approach to identify biomarkers for IEMs, articulated by Duran et al. (1982), as the "continuous search for 'new' metabolites [that] may eventually lead to a better understanding of the relationship between the clinical conditions and their biochemical abnormalities". In the case of IVA, the identification of the 11 biomarkers summarized in Table 1 of Chapter 4 embraced metabolites obtained from research conducted over a period of 40 years – from the first abnormal metabolites for IVA identified using packed-GC columns (Tanaka et al., 1966) to the description of N-isovaleryl leucine identified through ESI-MS/MS analysis (Loots et al., 2005). These markers were traditionally identified by extensive and time-consuming analytical procedures and based on samples from a single or a few individuals. In contrast, by using contemporary high resolution instrumentation and bioinformatics analysis, the metabolomics approach revealed an extensive complement of metabolites in biological samples, illustrating the holistic nature of metabolite profiles. The availability of technologically highly sensitive analytical equipment [e.g. NMR and 'hyphenated'-MS platforms (GC, LC or CE linked to various MS configurations, e.g. TOF-MS, QQQ or MS\(^n\)], software for spectral analysis (e.g. AMDIS or CHROMATOF for deconvolution) and several commercial (e.g. AMDIS and NIST) and customised metabolite libraries for metabolite identification lend substantial impetus to innovative ideas for further development and refinements in predictive laboratory medicine, as described below.

- **The context of systems biology**

Systems biology has been defined by Hood et al. (2004) as "...the scientific discipline that endeavours to quantify all of the molecular elements of a biological system to assess their interactions and to integrate that information into a comprehensive profile that serves as predictive hypotheses to explain emergent behaviours". With

---

\(^2\) Reduced metabolomics data refers to data from which either or both outlier features or cases have been removed, often by application of a standardized outlier removing bioinformatics technique, like a Mahalanobis analysis (Hadi, 1992).

\(^3\) Normalization of metabolomics data means an approach of adjusting values measured on different scales to a notionally common scale, e.g. the expression of metabolite concentrations in this thesis adjusted to the creatinine concentration in the urine samples (De Livera et al., 2012).
this in mind, the global metabolite profile of the IVA model revealed biomarkers for the disease and secondly, indicated significant metabolic changes which strongly resemble the systems approach of the definition. Consequently, the unique potential of metabolomics and its use in IEMs is clearly illustrated through our study of IVA.

In this regard, a model of secondary metabolic pathways and the involvement of different cellular compartments was identified during this study (Chapter 4, Fig. 4) which subsequently contributes to the understanding of the IVA biological system. Indeed, the statistically significant presence of 2-hydroxyisovaleric acid in untreated IVA patients points to the involvement of peroxisomes with phytanoyl-CoA hydroxylase as the most likely enzyme involved. More importantly, primary mitochondrial dysfunction was evident through the absence and presence of free carnitine and carnitine conjugation in untreated and treated patients, respectively. Consequently, detoxification pathways, involving carnitine conjugation and glycine acylation, were shown to serve as important "rescue" mechanisms which enable IVA patients to be treated. The carbohydrate-related biomarkers identified, mostly due to the therapeutic diet, are also described in Chapters 3 and 4. In addition, pathophysiological aberrations (discussed in Chapter 5) as well as possible nutritional deficiencies in treated IVA patients due to the dietary regimen (addressed in Chapter 7), was also disclosed. In summary, the results obtained with the use of metabolomics as a data-driven, informative and even predictive tool supports the initial aim that was set for this aspect of the thesis.

Evidently, future prospects and potential investigations should be kept in mind if IVA is studied (as well as other IEMs) in relation to systems biology. The induced secondary pathways and associated metabolites (as shown in Chapter 1, Fig. 2) brought about by the IVD deficiency, may lead to the discovery of new pathophysiological biochemical mechanisms which in turn uniquely affect the homeostasis in the cells of the individual IVA patient. An example of the existing as well as potential role of IVA metabolites in a biological system is comprehensively portrayed in Fig. 3, which illustrates the effect of accumulating isovaleryl-CoA and isovaleric acid (as a secondary product formed by a yet to be identified thioesterase) in the cell. This thesis has addressed some of the effects (marked in red in the figure) on the cell, but it may well be that novel avenues (correspondingly marked in blue) should be investigated in vitro as well as in vivo, which subsequently may lead to greater understanding of phenotypical variation within IVA groups.
In addition, various catabolic intermediates of the leucine pathway (Chapter 1, Fig. 1) as well as IVA-related metabolites (Chapter 1, Fig. 2) have been implicated in signalling pathways. Leucine and to a lesser extent α-ketoisocaproic acid, act as secondary regulators of glucose utilization (especially in the muscle and brain), lipid and cholesterol metabolism, protein anabolism and the inflammatory response in adipose tissue, among other functions (Gao et al., 2003; Lynch et al., 2003, Yoshizawa, 2004; Macotela et al., 2011; Su et al., 2012). Furthermore, the presence of metabolic decompensation in IVA leads to the subsequent activation of a catabolic state associated with hypoglycemia and elevated cellular AMP. This pathophysiological effect triggers a cascade of several signal transduction pathways (e.g. activation of AMPK and mTORC) (Howell and Manning, 2011; Laplante and Sabatini, 2012) that tend to induce recovery of cellular homeostasis.

A further consequence of a catabolic state which occurs in IVA patients, is proteolysis. It has been shown that leucine and more importantly isovalerylcarmitine (elevated in IVA patients) may regulate proteolysis in the liver (Miotto et al., 1998). The latter may play a role as an anti-proteolytic agent, through signalling pathways, during acute metabolic decompensation. In addition, the presence of 3-hydroxyisovaleric acid (elevated in some IVA patients) has been shown to play an important role in several signal transduction mechanisms, which indicates its multifaceted function in biological systems (Eley et al., 2007; Pimentel et al., 2011; Wilson et al., 2008). Moreover, certain PUFAs, e.g. EPA (found to be reduced in IVA patients undergoing treatment; Chapter 7) have been shown to be linked to signal transduction processes, important in protein synthesis (Whitehouse et al., 2001; Eley et al., 2007). In conclusion, these metabolic variations and mechanisms should be
taken into account from a systems biology perspective and merit attention in the future investigation of the pathophysiology of disease. These complex cellular mechanisms have to our knowledge not been investigated in IVA specifically and may help to explain the phenotypical variation in this disease.

• New biochemical insights

The metabolomics approach was instrumental in providing new biochemical insights with regard to untreated and treated IVA patients. Several pathophysiological and therapeutic features of this specific IVA cohort were identified through metabolomics and prompted further investigations. Consequently, the final aim of this thesis was to focus on one pathophysiological aberration i.e. hyperammonemia, as well as the nutritional status of treated IVA patients.

The metabolomics finding of a significant elevation of N-isovalerylglutamate in the urine of IVA patients coincided with our study of the mechanism of secondary hyperammonemia and the consequent formation of N-isovalerylglutamate in IVA. To this end, an in vitro determination of the activity of N-acetylglutamate synthase (NAGS), one of the vital steps in the urea cycle which plays a key role in ammonia elimination, was developed (Chapter 5). Subsequently the mechanism of secondary hyperammonemia present in IVA and several other short-chain and short/branched chain organic acidemias was studied. Short-chain and short/branched chain acyl-CoAs, including isovaleryl-CoA, had various effects as substrates for and inhibitors of NAGS, as described in Chapter 5. Consequently, the inhibition of NAGS was found to be a contributing factor to secondary hyperammonemia, as described in Chapter 5.

The final finding of this in vitro investigation of secondary hyperammonemia in IVA and other short-chain related organic acidemias did, however, raise additional questions concerning the factors responsible for the secondary hyperammonemia. Several contributing mechanisms on a biochemical level may include:

• The varied substrate specificity of several acyl-CoA dehydrogenases and acylases, which may limit accumulation of acyl-CoAs.

• The availability of acetyl-CoA and free CoA in the mitochondrion.

• The potential variation in the activity of urea cycle enzymes in individual patients.

• The activation of secondary and detoxification pathways, which consequently eliminate accumulated acyl-CoAs.

• The subsequent effect (activation or inhibition) of alternative N-acylglutamate conjugates on carbamyl phosphate synthetase 1 (CPS).
In conclusion, the function of the urea cycle which is potentially influenced by described biochemical variation should be considered in the treatment of hyperammonemia. Consequently, the benefits of the administration of pharmaceutical agents such as N-carbamylglutamate, may be of use in certain patients who are more prone to attack of acute hyperammonemia, as discussed in Chapter 5. These considerations in treatment of pathophysiological effects, for example hyperammonemia, again emphasize a need for a personalized approach to treatment of IEMs.

The study of NAGS inhibition led to the development of an improved UPLC-MS/MS activity assay applicable to a small amount of liver homogenates obtained by needle biopsy as described in Chapter 6. The measurement of NAGS for primary as well as secondary NAGS deficiencies (due to organic acidemias and drug-induced incidents) has proved to be problematic as explained in Chapter 6. We succeeded in setting up a reproducible and sensitive NAGS assay in liver tissue to investigate hyperammonemia on an enzymatic level, thereby contributing to the identification of patients having a primary NAGS disorder and in addition being able to investigate secondary hyperammonemia as a pathophysiological complication.

Finally, we addressed some aspects of the nutritional status of treated IVA patients. Chapter 1 section 4 emphasizes the importance of dietary adjustments and the advent of "detoxifiers" as well as therapeutic support of patients with IVA. In this regard, the IVA metabolomics study (Chapter 4) showed moderately increased methylmalonic acid in urine of treated IVA, which may be potentially associated with functional vitamin B12 deficiency (Herrmann et al., 2003). Consequently, functional vitamin B12 markers were measured but results in Chapter 7 showed no clear evidence of vitamin B12 deficiency on biochemical as well as symptomatic levels (e.g. absence of macrocytic anemia). We suggest that future studies, which include a larger cohort of treated patients as well as improved biochemical assessment of functional vitamin B12 status through the measurement of holotranscobalamin, as proposed by Heil et al. (2012), should be considered to confidently exclude a vitamin B12 deficiency. Furthermore, the inhibition of succinyl-CoA ligase by isovaleryl-CoA, subsequently resulting in elevated methylmalonic acid levels, has been reported by Berger et al. (1982) and consequently mimics a functional vitamin B12 deficiency. The latter is unlikely in the case of our study, for significantly elevated methylmalonic acid levels were observed in treated patients and not in untreated patients (Chapter 4). The influence of isovaleryl-CoA on succinyl-CoA ligase activity should however be reinvestigated to obtain a more convincing prospective.

The results in Chapter 7 irrefutably indicated an overwhelming omega-3- and omega-6 fatty acid depletion, which was attributed to dietary intervention. The
depletion of essential fatty acids can have a profound effect on the neurological development of a young child as well as possible immunological complications and need to be investigated further (Vlaardingerbroek et al., 2006). Moreover, the incorporation of accumulated isovaleryl-CoA into the fatty acid synthesis pathway has been mentioned shortly in a report by Malins et al. (1972), but was not investigated in our study. Consequently, it should be noted that limited production of essential fatty acids may be caused by the direct biochemical influence of isovaleryl-CoA on the fatty acid biosynthesis pathway and warrants further investigation. In summary, Chapter 7 reports the nutritional insufficiencies of treated IVA patients and therefore concludes that their nutritional status needs continuous monitoring of several essential biomolecules required for vital biochemical processes.

3. Future prospects: Towards predictive laboratory medicine

In general, current clinical and laboratory practices in Western society still focus predominantly on symptomology and/or a correlated diagnosis after the onset of disease, which are the foundation of specific therapies. To some degree, this approach has led to ever increasing healthcare costs, yet the prediction, precise diagnosis, prevention and treatment of both acute and chronic diseases remains incomprehensive. In addition, the limited understanding of the underlying mechanisms involved in the onset, progression and therapeutic intervention of many contemporary diseases has led to the growing awareness of a need to transform current diagnostic views in health and disease (Naylor and Chen, 2010). A special feature of a biological system is its diversity and corresponding variability. Personal medicine addresses this characteristic and is consequently paramount in our understanding of disease and the treatment of the individual patient at the appropriate time in a cost-effective manner (Zhang et al., 2012).

Furthermore, personalized medicine may bring about the understanding of pathophysiological effects on a molecular level and may even lead to the diagnosis of diseases or disorders prior to clinical onset. It ultimately seeks to cautiously predict diagnosis and consequently classify the illness in order to optimize therapy appropriate to a patient’s unique molecular profile and biological make-up. Current ideas on personalized medicine are thus not just enthusiastic views on innovations in future health-care, but aspire to investigate human complexity and variability through systems biology, a growing scientific movement leading to an integrated diagnostic and therapeutic approach (Naylor and Chen, 2010). Moreover, even within the realm of healthcare costs, Aspinall and Hamermesh in the Harvard Business Review (2007) recognized personalized medicine as a breakthrough of targeted therapies that could save many lives as well as a great deal of money.
Van der Greef et al. (2007) and Ginsburg and Willard (2009) suggest that personalized medicine forms part of an integrated, coordinated evidence-based approach within the context of systems biology which includes the use of current "-omics" technologies to understand disease in relation to health. This philosophy is also evident from the present study described in this thesis. A range of current and novel laboratory and diagnostic protocols, which are reproducible as well as repeatable are essential in order to understand several aspects of a biological system. This evidence-based approach is summarized in the model shown in Fig. 4. It is a more elaborate and sophisticated representation of the model presented in Chapter 1, Fig. 4, but it was also inspired by a related model proposed for a personalized approach to the treatment of cancer (Chin et al., 2011). The model emphasizes the following:

- The focus on the variability in IEMs, covering patients presenting with conditions ranging from acute symptoms (as in IVA) to chronic or virtually asymptomatic states and/or a characteristic phenotype (as described by Ensenauer et al., 2004). This pipeline, however, still retains the important general protocol followed for the diagnosis of IEMs.

- The requirement of different interventions and treatment regimens: emergent symptomatic interventions in patients presenting with acute symptoms (often in intensive care) towards well-founded and rationalized sophisticated interventions, including treatment and monitoring of progress.

- The importance of input from clinicians (especially in acute cases), but also the use of more specialized sources and protocols such as whole genome sequencing (WGS), whole exome sequencing (WES) and metabolomics are expected to define the resources required for the clinical and analytical pipeline as well as the costs involved in implementing the sophisticated mode of personalized health.

Despite the medical advances which continue up to the present time, numerous disease states can be explained only by complex "traits" rather than by alteration of a single gene, gene product or metabolite (Dipple and McCabe, 2000). Thus, in order to gather a more thorough and relevant understanding of disease, one must obtain a comprehensive perspective of the biological system, thereby uncovering the interdependent and dynamic pathway, network and cellular events that undergo change, resulting in disease predisposition, onset and progression. Hence, as noted recently by Lemberger, the application of systems biology to human biology and medicine should provide "...a deeper understanding of the genotype–phenotype relationship; impact of the interactions between environmental conditions and genotype; novel mechanistic and functional insights based on global unbiased..."
approaches; and elaboration of powerful predictive models capturing the intricacies of physiological states" (Lemberger, 2007; Naylor and Chen, 2010). Application of the clinical-analytical pipeline (Fig. 4) could hopefully contribute to this deeper understanding of the genotype–phenotype relationship in inherited diseases.
Fig. 4: A putative model describing the route towards improved personalized medicine. The proposed model indicates the two major research areas which include traditional diagnostic protocols (to the left side of the pipeline) as well as improved and novel biochemical and bioinformatic applications (middle and right side of the pipeline). The lower panel indicates possible points of entry in the clinical and analytical pipeline, and the upper panel presents possible modes of diagnosis, treatment or other interventions. Routine diagnosis through targeted metabolite identification, enzymatic and IVD gene specific analyses (Chapter 2) as well as therapeutic intervention including nutritional insufficiencies (Chapter 7) were addressed in this study. In addition, the use of metabolomics (Chapter 3 and 4) and the investigation of the pathophysiological mechanism of hyperammonemia (Chapter 5) further illustrate the importance of assessing disease in a biological system. The research done on IVA emphasizes that further scientific exploration is ultimately needed to improve the quality of life in treatable IEMs and consequently leads towards predictive laboratory medicine.
It is thus suggested that three research settings are required to potentially understand the phenotypical variation in IVA and to fully address personalized medicine. The use of untargeted metabolomics as well as proteomics may point to the involvement of secondary pathways and pathophysiological mechanisms including changes in signalling pathways (Collins et al., 2006). The application of next-generation sequencing (NGS), including WES (Yang et al., 2013) and/or WGS (Brunham and Hayden, 2012), may well become paramount in exploring phenotypical variation of IVA. The latter may lead to the identification of polymorphisms, consequently resulting in “inadequate” enzyme expression and function, which are involved in secondary pathways in individual IVA patients. Lastly, an IVA animal model may provide various biological samples, in which pathological mechanism of IEMs may be investigated by "-omics" technologies (Wajner and Goodman, 2011). However, the development of such an animal model for IVA is not yet available for these comprehensive studies (personal communication with Professor J. Vockley, and Professor R. Ensenauer). In summary, results obtained from this work may yield new options for investigating IVA and help to resolve unexplained phenotypic variations as observed in the patients and thereby contribute to the development of personalized medicine to use in everyday healthcare in the future.

In conclusion, given the lack of genotype-phenotype correlation for single gene disorders, personalized medicine is clearly gaining importance in the field of IEMs. In this regard, as a biochemist, I can only endorse the view expressed in the Presidential Address at the opening of the 11th International Congress of Inborn Errors of Metabolism in 2010 (McCabe, 2010). "The metabolome is our world. We have explored and exploited the metabolome to identify new diseases, to examine disease pathogenesis, to treat metabolic diseases, and to develop and expand NBS. ...... Those investigators who are focused on genomics, transcriptomics and proteomics are seeking to move toward the metabolome". The integrated approach followed in this thesis, even by using a well-studied disease such as IVA as model, clearly emphasized the important inputs that we as biochemists could make towards predictive laboratory medicine: as experts in the field of metabolism, in understanding and interpretation of metabolomics data on a systems level and as members of a multidisciplinary team of professionals, committed to the well-being of patients suffering from an IEM.

References


General discussion and future prospects


Laplante M1, Sabatini DM. 2012. mTOR signaling in growth control and disease. Cell 149:274-93


