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# Amino Acids Profiling for the Diagnosis of Metabolic Disorders

*Yana Sandlers*

## Abstract

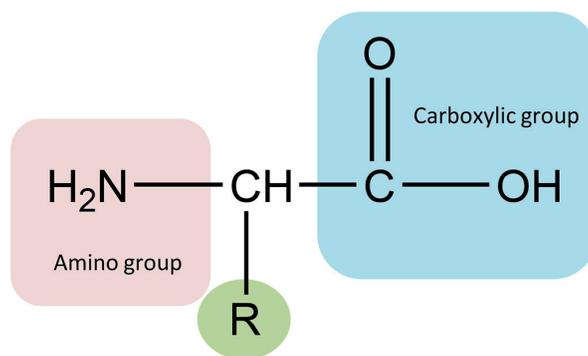
Inborn errors of metabolism (IEM) represent a group of inherited diseases in which genetic defect leads to the block on a metabolic pathway, resulting in a single enzyme dysfunction. As a downstream consequence of the residual or full loss of the enzymatic activity, there is an accumulation of toxic metabolites in the proximity of the metabolic block and/or a deficiency of an essential metabolic product which leads to the clinical presentation of the disease. While individually IEMs are rare, a collectively estimated incidence of metabolic inherited disorders is 1:800. The genetic basis of IEMs can involve abnormalities such as point mutations, deletions or insertions, or more complex genomic rearrangements. Categorization of IEM can be simply made on the basis of the affected metabolic network: fatty acids oxidation disorders, protein/amino acids metabolism disorders, disorders of carbohydrate metabolism, lysosomal storage diseases, peroxisomal disorders, and mitochondrial diseases. This chapter will overview amino acid metabolism-related inherited disorders and amino acid analysis for the diagnosis and routine monitoring of this category of IEMs.

**Keywords:** inborn error of metabolism, amino acids disorders, quantitative amino acids analysis, ion exchange chromatography, mass spectrometry

## 1. Introduction

Amino acids (**Figure 1**) play multiple important roles in our body: they are basic structural protein units and precursors of neurotransmitters, porphyrins, and nitric oxide. Furthermore, amino acids derived from the dietary proteins serve as energy source since while catabolized in our body, amino acids form organic acids that can replenish Krebs cycle and ammonia that eliminates through urea cycle [1].

Amino acids disorders (also called aminoacidopathies) are a group of inborn errors of metabolism diseases, caused by the inherited defects in pathways involved in amino acids metabolism. All primary amino acids disorders (**Table 1**) follow an autosomal recessive mode of inheritance which means that the mutation caused a metabolic block is present in the genetic material of both parents. As a result of mutation, the inherited defect is reflected downstream as a lack or a partial biological activity of enzymes involved in amino acids metabolism. Consequently, some substrates in these pathways accumulate or are diverted into alternative pathways. Therefore, amino acids disorders are biochemically characterized by abnormal levels of single or several amino acids and their downstream plasma and/or urine metabolites (**Tables 2–6**). Amino acid disorders are presented with variable and often nonspecific clinical symptoms. In conjunction with medical support, these disorders are managed by nutritional restrictions, supplements and medical



**Figure 1.** The general structure of amino acids consist of an amino group, a carboxylic group and a variable R side chain that has a major effect on solubility and polarity.

foods that limit consumption of an offending amino acid or in some cases protein consumption. It is important therefore routinely perform amino acids' analysis to monitor dietary treatment outcomes in already diagnosed individuals. In the next chapters, primary amino acid disorders are reviewed and quantitative amino acid analysis in clinical settings is discussed.

### 1.1 Phenylketonuria

Phenylketonuria (commonly known as PKU, incidence 1 in 13,500–19,000 births in the United States [2]) is an inherited disorder of phenylalanine metabolism characterized by phenylalanine hydroxylase deficiency (**Figure 1**). The enzyme catalyzes the conversion of phenylalanine to tyrosine in the presence of tetrahydrobiopterin (BH<sub>4</sub>) as a cofactor. Based on plasma phenylalanine level, PKU is categorized by severe (Phe > 1200 μmol/L), mild (Phe = 600–1200 μmol/L) and hyperphenylalaninemia (above the normal cut off but below 600 μmol/L) phenotypes. Clinically PKU can be presented with growth failure, global developmental delay, severe intellectual disabilities and other severe symptoms. During pregnancy, elevated levels of phenylalanine have teratogenic effects on the developing fetus [3] and the condition is recognized as maternal PKU. Phenylalanine accumulation is also seen in defects of biopterin cofactor biosynthesis and regeneration [4] (**Table 1**). Nutritional management of PKU targets excessive accumulation of phenylalanine by restriction of natural protein intake in combination with the use of phenylalanine-free protein substitutes.

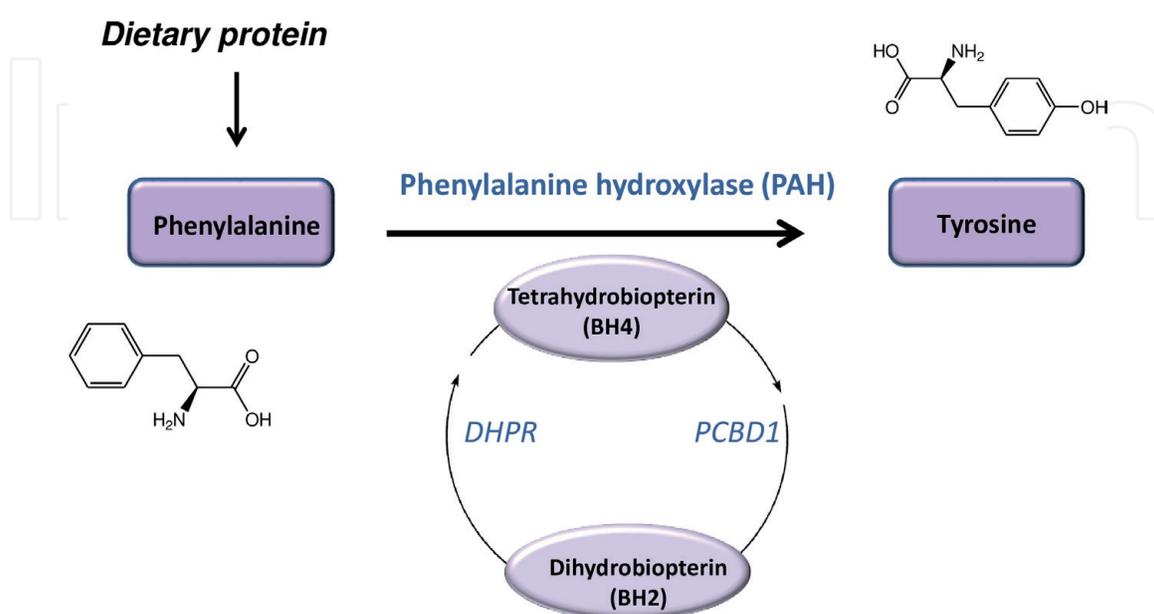
### 1.2 Disorders of tyrosine metabolism

Tyrosine metabolic pathway consists of five enzymatic reactions taking place mainly in hepatocytes and renal proximal tubules. Tyrosinemia I is the most severe inherited disorder of tyrosine metabolism caused by a deficiency of fumarylacetoacetate hydrolase, the last enzyme in the tyrosine catabolic pathway. The disorder has a high incidence in French Canadian ethnicity [5] and involves hepatorenal dysfunction. Tyrosinemia II is caused by a deficiency of the hepatic tyrosine aminotransferase and manifested by mental retardation and other severe symptoms [6]. A deficiency in the activity of 4-hydroxyphenylpyruvate dioxygenase leads to tyrosinemia III, a rare disorder characterized by mild mental retardation and/or convulsions [7, 8]. All three disorders biochemically characterized by high levels of plasma tyrosine (hypertyrosinemia) and urine excretion of downstream tyrosine metabolites (**Table 1**). Elevated plasma tyrosine can also be seen due to vitamin-C responsive transient tyrosinemia during the neonatal period (**Figure 2**).

Aromatic amino acids disorders			
Disorder name	Amino acid involved	Enzyme or transport defect	Additional biomarkers
PKU classical	Phe (B) high	PAH	Phe: Tyr ratio (B), phenylpyruvic, phenyllactic and 2-hydroxyphenylacetic acids (U)
Defect of bipterin cofactor biosynthesis	Phe (B) high	GTPCH	Low bipterin, neopterin (U)
Defect of bipterin cofactor biosynthesis	Phe (B) high	PTPS	Low bipterin, high neopterin (U)
Defect of bipterin cofactor regeneration	Phe (B) high	PCBD1	High neopterin and primapterin (U)
Defect of bipterin cofactor regeneration	Phe (B) high	DHPR	High bipterin (U) and low DHPR activity in dried blood spots
Tyrosinemia I	Tyr (B) high	FAH	Succinylacetone (DBS, U), 4-hydroxy-phenylpyruvic, 4-hydroxy-phenyllactic acids (U)
Tyrosinemia II	Tyr (B) high	TAT	4-hydroxyphenylpyruvic, 4-hydroxy-phenyllactic acids (U)
Tyrosinemia III	Tyr (B) high	HPPD	4-hydroxyphenylpyruvic, 4-hydroxyphenyllactic acids (U)

PAH, phenylalanine hydroxylase; GTPCH, GTP cyclohydrolase; PTPS, 6-pyrovoyltetrahydropterin synthase; PCBD1, pterin-4 $\alpha$ -carbinolamine dehydratase; DHPR, dihydropterin reductase; FAH, fumarylacetoacetate hydrolase; TAT, tyrosine aminotransferase; HPPD, 4-hydroxyphenylpyruvate dioxygenase.

**Table 1.**  
 Laboratory findings in aromatic amino acids disorders.



**Figure 2.**  
 Reaction catalyzed by phenylalanine hydroxylase. Tetrahydrobiopterin (BH<sub>4</sub>) is a co-factor of PAH. DHPR, dihydropteridine reductase; PCBD1, pterin-4- $\alpha$ -carbinolamine dehydratase.

### 1.3 Maple syrup urine disease

Maple syrup urine disease is a disorder of branch chain amino acids metabolism caused by a deficiency of branched-chain  $\alpha$ -keto acid dehydrogenase complex. MSUD is presented with five clinical phenotypes on the basis of the age at onset, the severity of symptoms and response to thiamine supplementation [9]. MSUD characterized biochemically by elevated plasma branched-chain amino acids (leucine, isoleucine, valine, allo-isoleucine) and their abnormal ratio (normal ratio is valine:isoleucine:leucine/3.5:1:2). The disease is managed by dietary leucine restriction, thus all branch chain amino acids and allo-isoleucine are routinely monitored. The classic MSUD is the most severe form of the disease characterized by no or very low residual enzyme activity and clinically manifested by developmental and neurological delays, encephalopathy, feeding problems, and a characteristic maple syrup odor in urine.

Disorders branched-chain amino acids			
Disorder name	Amino acid involved	Enzyme or transport defect	Additional biomarkers
MSUD	BCAA (B) high, allo-Ile (B) high	BCKDC	Plasma ratio of Val:Ile:Leu (3.5:1:2), branch chain 2-ketoacids and 2-hydroxyacids (U)

*BCKDC, branched-chain ketoacid dehydrogenase complex.*

**Table 2.**  
*Laboratory findings in MSUD.*

### 1.4 Urea cycle disorders

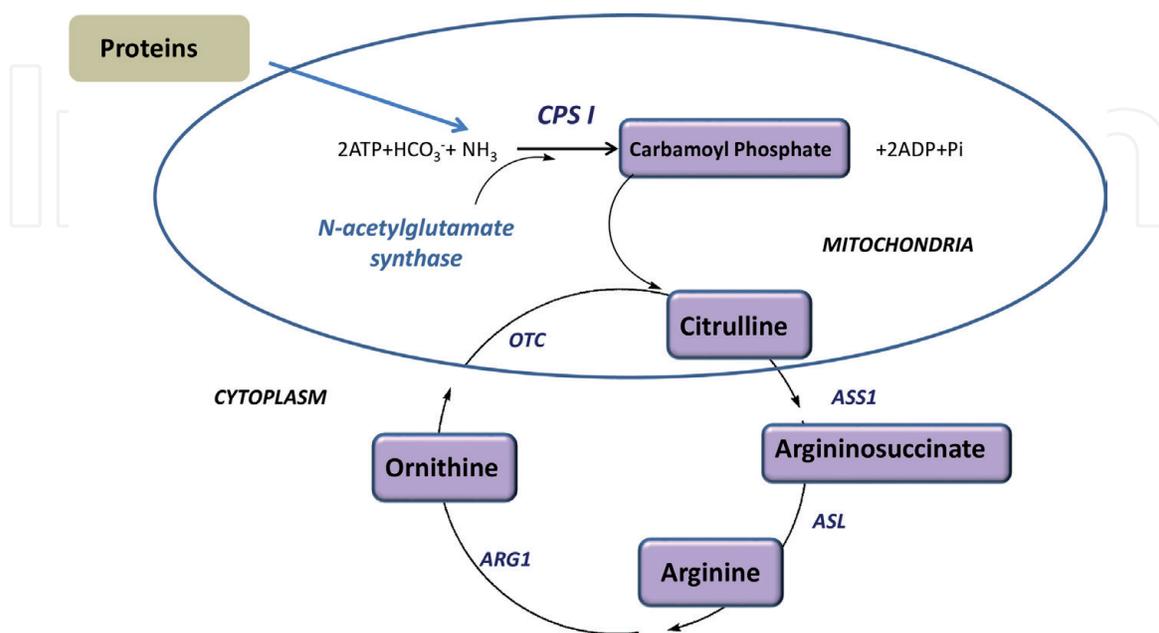
During protein catabolism, amino acids' carbon skeleton is metabolized to gluconeogenic and/or ketogenic precursors whereas nitrogen group is converted to ammonia through the deamination process. Toxic ammonia derived from amino acids and other metabolic sources is entering the urea cycle and further is converted to the readily excreted and nontoxic urea. The cycle takes place in the liver and a deficiency of any enzymes or transporters involved in the urea cycle can cause ammonia accumulation (hyperammonemia) which has a highly toxic effect on the central nervous system. The overall estimated incidence of urea cycle disorders is 1:8000. All urea cycle disorders have an autosomal recessive inheritance, with the exception of ornithine-transcarbamylase deficiency (OTCD), which is X-linked. Plasma citrulline is a key amino acid in the biochemical diagnosis of urea cycle defects (**Table 3**).

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome is caused by the mutations in the SLC25A15 or ORNT1 gene which result in the deficiency of ornithine translocase. The protein transports ornithine, lysine, and arginine across the inner mitochondrial membrane in peripheral tissues and pericentral hepatocytes. ORNT1 deficiency reduces the availability of mitochondrial ornithine, which leads to the ornithine increase in the cytosol (hyperornithinemia). In the liver, since the mitochondrial ornithine is a required substrate for ornithine transcarbamylase (OTC), the reduced level of mitochondrial ornithine slows down flux through the urea cycle (**Figure 3**). As a result of the reduced capacity of the urea cycle, ammonia and carbamoyl-phosphate levels increase (hyperammonemia). At the same time, an excess of carbamoyl-phosphate is diverted to react with lysine to form homocitrulline (homocitrullinuria) or enters in the pyrimidine pathway, to form orotic acid which is later excreted in urine. Similarly, as for other urea cycle disorders, early diagnosis in infancy may improve the clinical outcome of HHH.

Urea cycle disorders			
Disorder name	Amino acid involved	Enzyme or transport defect	Additional biomarkers
Citrullinemia I	Cit (B) markedly elevated	ASS	Hyperammonemia, orotic acid (U), can be accompanied by high glutamine and alanine (B)
Citrullinemia II/citrin deficiency	Cit (B) moderate high, Met, Lys (B)	Asp/Glu mitochondrial exchanger	Hyperammonemia, orotic acid (U). Citrulline is moderately elevated
CPS-I deficiency	Cit (B) low	CPS1	Can be accompanied by high glutamine and alanine (B)
Ornithine transcarbamylase deficiency	Cit (B) low	OTC	Nonspecific amino acid profile: increased glutamine, alanine and decreased ornithine, arginine (B). Orotic acid (U) markedly increased
Arginino-succinic acidemia	ASA (B), (U) elevated	ASL	Low citrulline, low arginine (B)
Argininemia	Arg (B) high	Arginase	Orotic acid (U), Normal or reduced citrulline (B)
HHH	Orn (B, U), high	ORC1	Homocitrulline (U) high
Co-factor producing N-acetyl glutamate synthetase deficiency	Gln (B) high	NAGS	Cit (B) low, alanine high (B)

*B, blood; U, urine.*

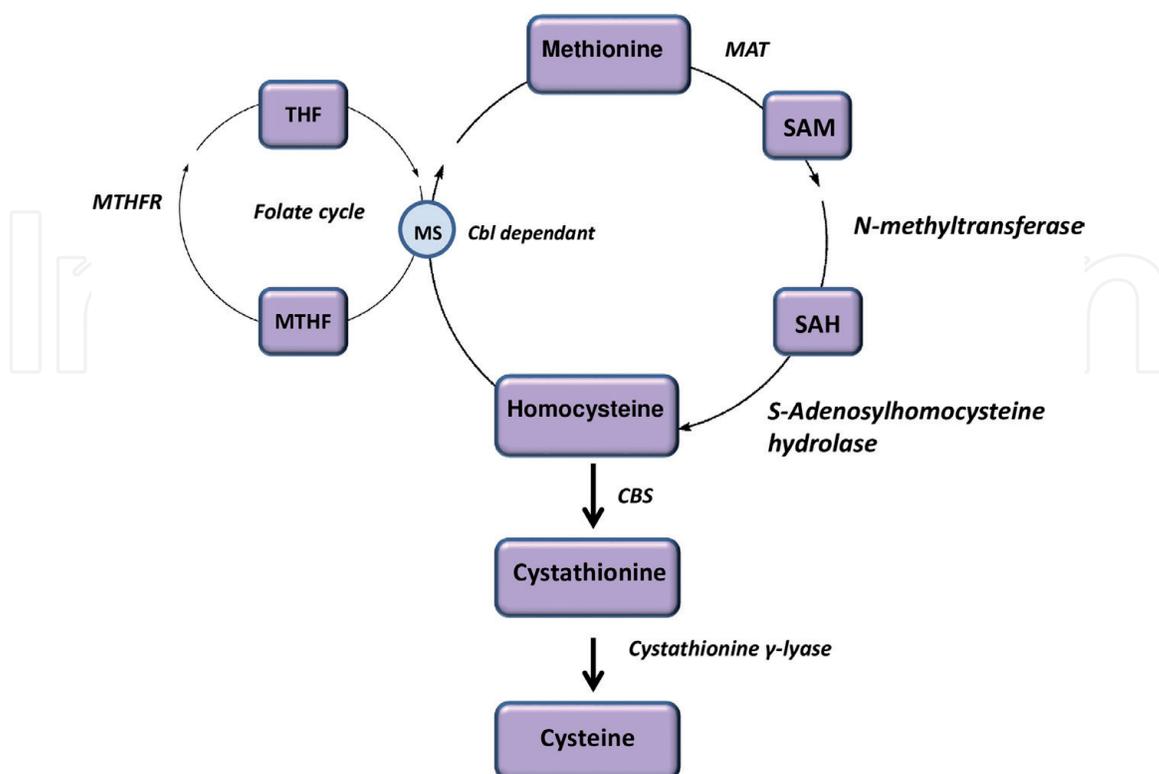
**Table 3.**  
 Laboratory findings in urea cycle disorders.



**Figure 3.**  
 Urea cycle. ARG, arginase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; CPS1, carbamoylphosphate I synthase; ORNT 1, mitochondrial ornithine transporter; OTC, ornithine transcarbamylase.

## 1.5 Disorders of sulfur amino acids metabolism

Homocystinuria is a disorder of methionine metabolism (**Figure 4**). The main biochemical finding in homocystinuria is accumulation of a sulfur-containing amino acid homocysteine and its metabolites in the blood and urine. Homocysteine is formed from methionine via transmethylation. Once generated homocysteine can be irreversibly degraded via transsulfuration pathway to cysteine or remethylated back to methionine by methionine synthase. Remethylation involves a transfer of methyl group from 5-methyltetrahydrofolate to homocysteine via cobalamin (Cbl)-dependent methionine synthase (MT) and links folate cycle and homocysteine pathway. Homocysteine can also be remethylated through an additional pathway which involves liver and kidney betaine-homocysteine methyltransferase. Defects in any of these steps can result in homocystinuria. The classic homocystinuria is caused by cystathionine  $\beta$ -synthase (CBS) deficiency [10], a key enzyme in the trans-sulfuration pathway that converts homocysteine into cystathionine. A block at cystathionine  $\beta$ -synthase limits transsulfuration to the cysteine and results in both increased homocysteine and methionine, the latter caused by enhanced remethylation. The remethylation homocystinuria disorders include methylene-tetrahydrofolate reductase deficiency (MTHFR) and defects of cobalamin (Cbl) metabolism [11]. It has to be noted that methionine and not homocysteine is analyzed through the newborn screening, thus, MTHFR disorder and the cobalamin defects may not be detected because methionine level in these disorders can be normal. To increase the detection rate in cobalamin related disorders and MTHFR, some studies report a benefit of adding total homocysteine analysis to the diagnostic workflow [12]. Total homocysteine is defined as the sum of all homocysteine species in plasma/serum, including free and protein-bound forms. The measurement of total homocysteine requires an immediate separation and freezing of the collected plasma.



**Figure 4.** Sulfur amino acids metabolism. CBS, cystathionine  $\beta$  synthase; Cbl, cobalamin; SAM, S-adenosyl methionine; SAH, S-adenosylhomocysteine; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; THF, tetrahydrofolate.

<b>Disorders of sulfur amino acids</b>			
<b>Disorder name</b>	<b>Amino acid involved</b>	<b>Enzyme or transport defect</b>	<b>Additional biomarkers</b>
Homocystenuria	Free homocystine (B, U) high	CBS	Total Hcy and methionine (B, U)
S-Adenosyl-homocysteine-hydroxylase deficiency	Met (B) high	AdoHcyas	Mildly elevated total plasma Hcy, S-adenosylhomocysteine, (B), S-adenosylmethionine (B)
Sulfate oxydase deficiency	SSC* (B, U) high	SUOX	Taurine, low cystine (B, U)
Molybden cofactor deficiency	SSC* (B, U) high	SUOX, XDH, AO	Taurine, low cystine (B, U), elevated hypoxanthine and xanthine (U), low uric acid (B)
Glycine-N-methyltransferase deficiency	Met (B) high	GNMT	S-adenosylmethionine (B)
Hyper-methioninemia	Met (B) high	MAT	

*CBS, cystathionine beta synthase; AdoHcyas, S-adenosylhomocysteine hydroxylase; SUOX, sulfate oxidase; XDH, xanthine dehydrogenase; AO, aldehyde oxidase; GNMT, glycine-N-methyltransferase.*  
 \*S-sulphocysteine may not be detectable in plasma using routine methods in sulfite oxidase and molybdenum co-factor deficiencies.

**Table 4.**  
 Laboratory findings in disorders of sulfur amino acids.

## 1.6 Nonketotic hyperglycemia

Nonketotic hyperglycemia (NKH) is a severe disorder of glycine metabolism. Glycine is catabolized through the four-peptide cleavage complex. P-protein, a pyridoxal phosphate-containing protein, T-protein, a protein required for the tetrahydrofolate-dependent reaction, H-protein, a protein that carries the amino-methyl intermediate and then hydrogen through the prosthetic lipoyl moiety, and L-protein, a lipoamide dehydrogenase. The disorder is so severe, that most of the affected individuals die within few months of life or survive with significant intellectual disabilities. Main laboratory findings in NKH is plasma and CSF elevated glycine.

<b>Disorders of amino acids transport</b>			
<b>Disorder name</b>	<b>Amino acid involved</b>	<b>Enzyme/transport defect</b>	<b>Additional biomarkers</b>
Cystinuria	Cystine (U) elevated	Cystine and dibasic amino acids in GI tract and renal tubule	Lysine, ornithine, arginine increase (U)
Lysinuric protein intolerance	Lys (U) markedly elevated	Cationic amino acids transporter SLC7A7	Arginine, ornithine moderate increase (U), orotic acid (U)
Fanconi syndrome	All amino acids elevated (U)	Defects in proximal renal tubule	

*U, urine.*

**Table 5.**  
 Laboratory findings in renal aminoacidurias.

Disorder of glycine metabolism			
Disorder name	Amino acid involved	Enzyme/transport defect	Additional biomarkers
Nonketotic hyperglycemia (NKH)	Gly (B, CSF) high	Mutations in Gly cleavage system	Increased CSF/plasma Gly ratio

*B, blood; CSF, cerebrospinal fluid.*

**Table 6.**  
*Laboratory findings in NKH.*

### 1.7 Renal aminoacidurias

Renal aminoacidurias are disorders have inherited defects that affect renal tubular reabsorption process. Thus these disorders are characterized by abnormal urinary amino acids.

## 2. Newborn screening

Early diagnosis may prevent serious implications of inborn errors of metabolism, including amino acids disorders and significantly decrease morbidity and mortality. Newborn screening is a public health program that facilitates early diagnosis by identifying neonates with potential treatable inborn errors of metabolism at the very early stages of their lives [13, 14]. This practice helps to manage the disease even for neonates that do not have evident symptoms in the first days of their lives. Amino acids analysis has always been an important part of the newborn screening. The first PKU screening bacterial inhibition assay was invented by Robert Guthrie in the early '60s [15]. Since that time, screening for IEMs is performed worldwide. In the United States, newborn screening is a state-mandated public health program ensuring that all newborns are screened for certain inherited conditions at birth. The panel of screening conditions varies from state to state. The advisory committee on heritable disorders in newborns and children advises the Secretary of Health and human services uniform screening panel, which currently consist of 34 core disorders and 26 secondary disorders. The recommended panel includes multiple amino acids related disorders (**Table 7**).

<b>Recommended uniform screening panel (RUSP)</b>	<b>Argininosuccinate aciduria, citrullinemia type I homocystinuria (cystathionine-<math>\beta</math>-synthase), maple syrup urine disease, phenylketonuria/hyperphenylalaninemia, tyrosinemia I</b>
Additional non-RUSP conditions	Nonketotic hyperglycinemia (NKH), prolinemia, hyperammonemia/ornithinemia/citrullinemia (HHH)
Secondary	Defects of bioprotein cofactor biosynthesis and regeneration, citrullinemia II, hypermethioninemia, tyrosinemia II, tyrosinemia III

**Table 7.**  
*List of amino acids disorders that are recommended by the Secretary of the Department of Health and Human Services (HHS) as a part of state universal newborn screening (NBS) program effective July 2018.*

## 3. Quantitative amino acids analysis

Quantitative amino acids analysis is an important tool for diagnosis of amino acids disorders and nutritional monitoring of individuals with already established diagnosis. Amino acids can be detected in most biological fluids, however, the most common

fluids for inborn errors of metabolism diagnostics and monitoring are blood, plasma, and urine. In some cases, cerebrospinal fluid (CSF) amino acid levels are also diagnostic (**Table 5**). Although each disorder is biochemically characterized by abnormal levels of a single or a few amino acids, quantitative a non-screening analysis, and interpretation is not restricted to those metabolites and consist from a panel of nearly 40 amino acids and specific ratios. For example, along with plasma phenylalanine level, it is important also to assess plasma phenylalanine/tyrosine ratio that can be used to differentiate between PKU and non-PKU hyperphenylalaninemia [16].

### **3.1 Factors affecting amino acid analysis**

The different chemical characteristics, a wide range of normal physiological levels [17–19], age groups variability and other factors detailed below represent a significant analytical challenge for the amino acid analysis. Diet is one of the significant factors that can highly affect amino acids levels [20, 21]. For example, meat and poultry consumption leads to increased excretion of  $\beta$  alanine and 1-methylhistidine. Thus blood collection intended for amino acids analysis is recommended after overnight fasting. Other factors such as urinary bacterial contamination can significantly alter urinary amino acids profile [22]. Some drugs interfere with amino acids metabolism [23] or cause signal artifacts. Valproic acid, for example, can cause an increase in plasma glycine. Anticoagulants used during sample collection also can contain interfering constituents [24]. For example, blood collection tubes containing sodium bisulfate in addition to heparin can yield a peak of S-sulfocysteine, falsely suggesting sulfite oxidase deficiency. Ethylenediaminetetraacetic acid (EDTA) additive in collection tube can produce ninhydrin-positive peaks, therefore lithium-heparin coated tubes are strongly preferred for the blood collection. An additional interfering factor to the amino acid analysis is a hemolysis as it may lead to the decrease of arginine with simultaneous increase of ornithine due to red blood cells arginase activity, and an increase in taurine that released from leukocytes and platelets. Serum is usually not a choice for the amino acids analysis, because blood needs to clot at room temperature during which asparagine is converted to aspartic acid and glutamine to glutamic acid.

For the urine analysis, a 24-h urine collection is preferred, alternatively, an overnight collection can be sufficient for the diagnostic purposes. In order to avoid artifacts, no preservatives are added to the urine sample.

Overall, during a prolong sample storage glutamine and asparagine decrease whereas glutamic and aspartic acids increase simultaneously. Additional markers of prolong storage are an increase of ethanolamine derived from phosphoethanolamine decomposition, increased tryptophan, GABA and taurine.

When cerebrospinal fluid is used for the analysis, it must be not contaminated with blood, as it leads to the nonspecific increase of multiple amino acids and can mask diagnostic findings.

Quantitative amino acids analysis implies in a variety of nonclinical fields such as biomedical research, bioengineering, food science, and agriculture. Multiple analytical methods have been developed over the years, however, some of these methods are not cost effective and labor intensive and thus are not applicable in clinical settings. The aim of next paragraphs is to describe the most common and widely used platforms in laboratory medicine field.

### **3.2 Ion exchange chromatography coupled with optical detection**

In early 50s, diagnostic quantitative amino acid analysis became feasible with Moore and Stein publication on plasma amino acids separation with polystyrene

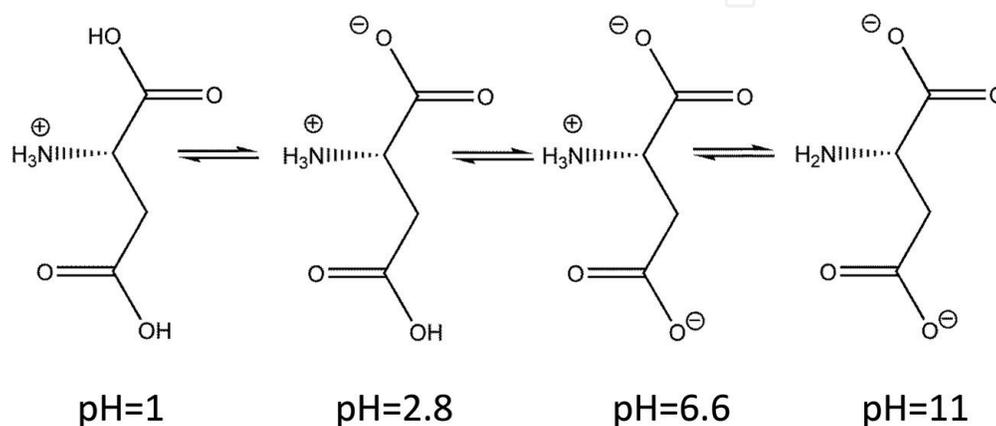
resin column [25] and the subsequent automatization of the technique [26]. The principle, called ion exchange chromatography (IEC) with a post-column derivatization, for a long time remained a gold standard for the clinical amino acids analysis. Nowadays, despite the methodological advancements, the ion exchange chromatography using a lithium buffer system, followed the post-column derivatization with ninhydrin and UV detection is still widely used in clinical setting.

Standard sample preparation for IEC amino acid analysis involves deproteinization with 35% (w/v) sulfosalicylic acid (SSA) added to the biological fluid. It is recommended to use one volume of SSA to 10 volumes of plasma. A fixed amount of non-physiological amino acid as an internal standard is added to all samples. Commonly used internal standards are D-glucosaminic acid, S-2-aminoethyl-1-cysteine, norvaline, and norleucine, however, norleucine can interfere with argininosuccinic acid peak. After a short incubation, centrifugation and filtration, the sample is ready for the injection and separation.

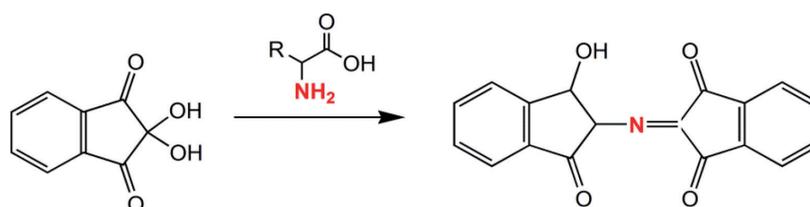
In IEC, the separation is driven by the ionic interactions between the amino acid and functional ligands linked to the stationary phase of the column. The chromatographic column is filled with negatively charged resins. The sample is loaded on the column in low acidic pH and at this point, all amino acids bear a positive charge and strongly interact with the column. Manipulation with a lithium buffer composition during the run alters pH and salt composition, and as a result, there is a change in amino acid charge status (**Figure 5**). As the isoelectric point is reached amino acid is not charged anymore and has weak interactions with the charged column.

The complex separation of multiple amino acids is achieved based on ionic interactions strength. Amino acids with the weakest ionic interactions to the column start to elute first. After column elution, amino acids are mixed with a post-column reagent and are optically detected. The most common and well-established post-column derivatization is reaction with ninhydrin that produces a purple Ruhemann's chromophore ( $\lambda_{\max} = 570 \text{ nm}$ , **Figure 6**) for  $\alpha$ -amino acids and yellow product with secondary amines ( $\lambda_{\max} = 440 \text{ nm}$ ) for such as proline and hydroxyproline [27].

The absorbance intensity of the produced colorful analyte originated from every eluted amino acid is proportional to the amino acid's concentration in the examined biological fluid. Despite the fact that IEC amino acids technique is highly reproducible with a good linearity over a broad range, it suffers from a long run time for the full amino acids profile (about 150 min), and a lack of specificity as amino acids identification is based solely on retention time. Furthermore, co-elution of some amino acids on standard IEC method is



**Figure 5.**  
Aspartic acid charge in different pH.



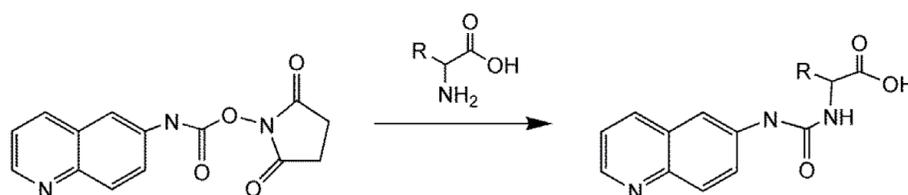
**Figure 6.**  
*Ninhydrin reaction with amino acid to produce Ruhemann's purple.*

observed. For example, homocitrulline co-elutes with methionine and make challenging HHH syndrome. Moreover, allo-isoleucine, a diagnostic marker for MSUD co-elutes with cystathionine. Argininosuccinic acid that accumulates in patients with argininosuccinate lyase deficiency has the same retention time as leucine. Additional drawback of the methodology is a limited stability of ninhydrin (recommended storage of the working solution  $\leq 1$  month) which adds up to the cost of the analysis.

### 3.3 RP-HPLC and RP-UPLC techniques

In recent years, reverse-phase high-performance liquid chromatography (RP-HPLC) and ultra-high performance liquid chromatography (UPLC) methods emerged as an alternative to the ion exchange chromatography. In RP-HPLC methods, the separation is based on hydrophobic interactions between the analyzed amino acid in the mobile phase and the immobilized hydrophobic ligands attached to the nonpolar column stationary phase. RP-HPLC offers a great resolution of very closely related molecules under a wide range of chromatographic conditions. For the optical detection, derivatization with o-phthalaldehyde (OPA) can be used as a pre-column or a post-column reaction. During the reaction, in the presence of thiol such as 2-mercaptoethanol, a stable fluorescent product is produced and can be detected by fluorimetry (excitation 340 nm and emission 410 nm) or UV (340 nm) [28, 29]. Although reproducible and automated [30], OPA derivatization method is not a good choice for proline/hydroxyproline and sulfur-containing amino acids detection. Alternative reagents for RP-HPLC with pre-column derivatization are phenylisothiocyanate (PITC, Pico-Tag commercialized by Waters) [31], dimethylamino-azobenzenesulfonyl-chloride (DABS-Cl) [32] and 9-fluorenylmethylchloroformate (FMOC-Cl) [33].

More advanced UPLC systems employ a small particle size (typically 1.7  $\mu\text{M}$ ) and a high pH range stable columns. These systems use less solvent and are operated in a high pressure which allows an excellent resolution achieved in a short time frame and thus potentially decreases turnaround time per sample. Narayan et al. analyzed 170 patient samples by pre-column 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatization (**Figure 7**) followed by reverse phase UPLC [34] and compared amino acids data to the traditional amino acids analyzer operated through ion



**Figure 7.**  
*AQC reaction with amino acid.*

exchange chromatography method. The study found that UPLC method is comparable to the reference IEC and thus adaptable to the clinical laboratory.

Peake et al. developed a modified RP-UPLC method and achieved a better resolution for tyrosine, glycine, arginine and homocitrulline peaks [35]. The improved method also provides enhanced resolution to separate ornithine from mesocystathionine. There is a high clinical significance to accurate ornithine analysis as ornithine's levels are diagnostic for hyperornithinemia-hyperammonemia-homocitrullinemia (HHH) syndrome. The developed UPLC method has several advantages. Due to the short analysis time, it is feasible to include calibration prior to the analysis of urgent samples with a special turnaround times. Overall, RP-UPLC decreases turnaround time per sample, however, commercial kit components have a very limited shelf life and thus the method is not cost effective for clinical laboratories with a small samples volume.

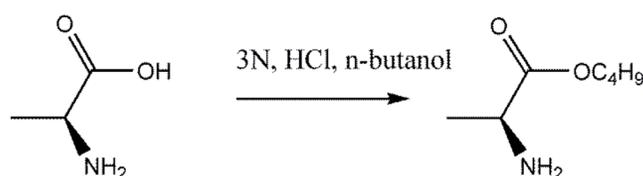
Overall, ion exchange chromatography, RP-HPLC, and RP-UPLC techniques have a good reproducibility and a high sensitivity in the low picomole range, however, they all are carried out with optical detection. The main drawback of this type of detection is a lack of specificity as amino acids identification is solely based on the retention time. This can potentially cause to the false findings. For example, in a standard ion exchange chromatography method, ampicillin and amoxicillin co-elute with phenylalanine and it can be reported as falsely elevated.

### 3.4 Flow infusion tandem mass spectrometry (FIA-MS/MS)

More recently, developments and advancements in mass spectrometry field led to the inclusion of tandem mass spectrometry (MS/MS) as an alternative high throughput and specific technique for the amino acids analysis. It is also feasible to separate amino acids by liquid chromatography prior to the mass spectrometry analysis, however, it is time-consuming in clinical settings. Instead, tandem mass spectrometry scans are used for the high throughput, cost-effective amino acids analysis. It has to be noted, that FIA-MS/MS is a screening analysis that widely implemented through the newborn screening initiative.

For the newborn screening, blood samples are typically collected on filter paper and a defined size (typically 3 mm) disks are punched out of the paper and are extracted. The early assays required derivatization by butylation (**Figure 8**) in order to improve detection limits and minimize ion suppression effects in a complex biological matrix. Currently, to increase a throughput, some clinical laboratories skip on the derivatization step. Extracted and derivatized samples are directly introduced by injection to the mass spectrometer instrument with no chromatographic separation. Usually, 5–10  $\mu\text{l}$  of a sample is injected into a flowing solvent at a very low (20–50  $\mu\text{l}$ ) flow rate. All screened amino acids (**Table 8**) are eluting at the same time whereas a typical run time is 1.5–2 min per sample. Every analyzed amino acid is assayed with the corresponding stable isotopic labeled standard.

The isotopic-labeled standards are closely related to the structure of the analyzed amino acids and have similar physicochemical properties to the target amino acids, but can be distinguished by mass spectrometry as they have a different



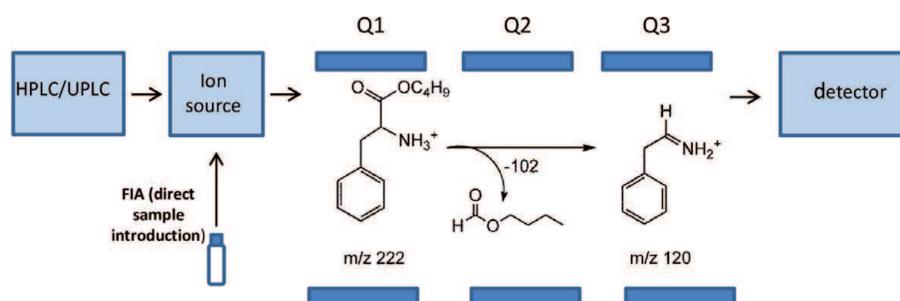
**Figure 8.**  
Derivatization of alanine with *n*-butanol.

Target amino acid	<i>m/z</i>	Internal standard	<i>m/z</i>
Alanine	146.1	<sup>2</sup> H <sub>4</sub> alanine	150.1
Arginine	231.2	<sup>13</sup> C, <sup>2</sup> H <sub>4</sub> arginine	236.2
Aspartic acid	246.2	<sup>2</sup> H <sub>3</sub> aspartic acid	249.2
Citrulline	232.2	<sup>2</sup> H <sub>2</sub> citrulline	234.2
Glutamic acid	260.2	<sup>2</sup> H <sub>3</sub> glutamic acid	263.2
Glycine	132.1	<sup>15</sup> N, <sup>13</sup> C glycine	134.1
Leucine/isoleucine	188.2	<sup>2</sup> h <sub>3</sub> leucine	191.2
Methionine	206.2	<sup>2</sup> H <sub>3</sub> methionine	209.2
Ornithine	189.2	<sup>2</sup> H <sub>2</sub> ornithine	191.2
Phenylalanine	222.2	<sup>13</sup> C <sub>6</sub> phenylalanine	228.2
Tyrosine	238.2	<sup>13</sup> C <sub>6</sub> tyrosine	244.2
Valine	174.2	<sup>2</sup> H <sub>8</sub> valine	182.2

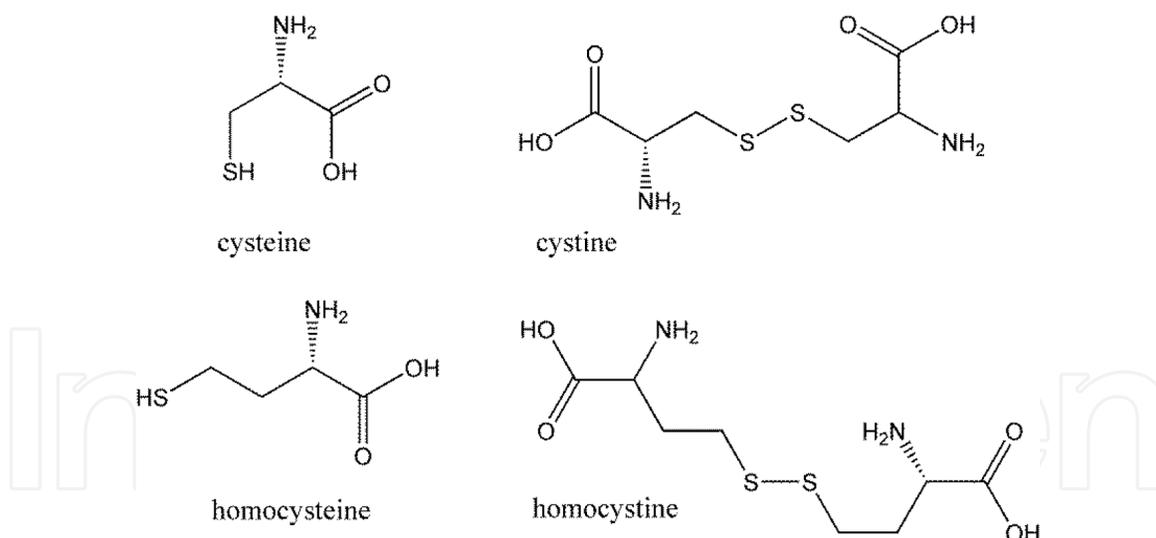
**Table 8.**  
 Amino acids analyzed by FIA-MS/MS for the standard newborn screening panel and their stable isotopic labeled internal standards.

mass to charge ratio (*m/z*) (Table 8). They are added at a known quantity, and the response of each analyzed amino acid is normalized by the response of the matching internal standard. This type of normalization reduces a systematic error due to the poor recovery and decreases multiple matrix effects. The inclusion of internal standards also corrects a batch to batch variability due to the sample preparation and overall raises the accuracy and precision of the assay.

The tandem mass spectrometer has five basic components: the ion source where all molecules are a subject to the soft ionization, a mass analyzer that separates analytes based on their mass to charge ratio (Q1), a collision cell where molecular ions encounter an inert gas and undergo fragmentation (Q2), a second mass analyzer to separate fragments produced in the collision cell (Q3), and a detector. In collision cell, most of the screened butylated  $\alpha$ -amino acids form a common and a very specific fragment of 102 Da (Figure 9). The tandem mass spectrometer then can be set to scan a constant mass difference of 102 Da and to produce a spectrum of the molecular ions derived from those amino acids that lost 102 Da in the collision cell (Q2) (Figure 9). Butylated amino acids with a basic side chains such as ornithine, citrulline loose ammonia and butyl formate in the



**Figure 9.**  
 Schematic presentation of tandem mass spectrometer. Phenylalanine (as butyl ester) loses 106 Da in the collision cell. When mass spectrometer operates in neutral loss scanning mode, it scans Q1 and Q3 in a synchronized manner. The mass difference of 102 Da (corresponds to a neutral fragment common to the most amino acids) passing through Q2 remains constant.



**Figure 10.**  
Sulfur amino acids and their disulfides.

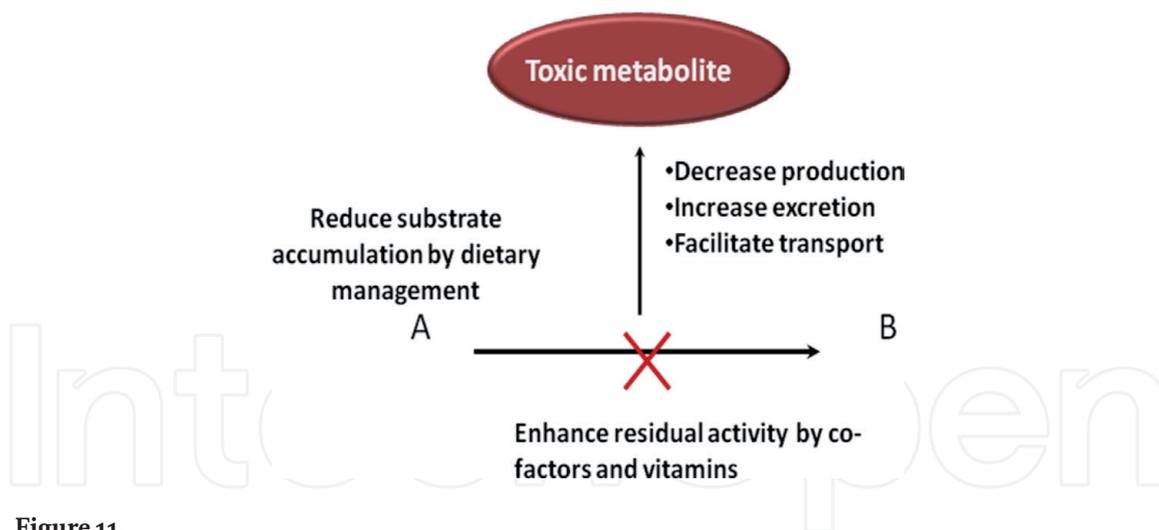
collision cell ( $m/z$  119). For glycine and arginine, the most intensive signal corresponds to the loss of 56 and 161 Da, respectively. All these specific losses or transitions can be detected by different and highly specific tandem mass spectrometer's scans in the single analysis.

The main limitation of the FIA-MS/MS is inability to differentiate amino acids that share the same  $m/z$  such as leucine/isoleucine and hydroxyproline (butylated derivatives  $m/z$  188), alanine/sarcosine (butylated derivatives  $m/z$  146) and in a more extended profiles glutamine/lysine (butylated derivatives  $m/z$  186), proline/asparagine (butylated derivatives  $m/z$  172). Also, FIA-MS/MS is not applicable for cysteine and homocysteine analysis since these amino acids are not stable and react to form cystine and homocystine (**Figure 10**). During the ionization process, cystine and homocystine produce double charged molecules and it complicates the analysis.

Due to the high sensitivity and selectivity, there are more mass spectrometry-based techniques available for the amino acids analysis, although because of extensive sample preparation or limited number of amino acids covered, these methods are not widely used in clinical laboratories. Gas chromatography mass spectrometry (GCMS) [36], capillary electrophoresis mass spectrometry (CEMS) [37], ion pairing (IP)-LC-MS/MS, HILIC-LC-mass spectrometry [38] and two column LC-MS/MS methods [39], ion pairing (IP)-LC-HRMS (TOF) [40] can be successfully applied for the physiological amino acids analysis although with some limitations.

#### 4. Diagnosis of amino acids related disorders

The initial diagnosis of amino acids disorders is based on clinical presentation and biochemical findings such as abnormal levels of specific amino acids (**Tables 1–6**) or accumulation of the downstream metabolites in biological fluids, however, these characteristics are very heterogenic and often nonspecific. The most common clinical indications for the quantitative amino acid analysis in neonates and pediatric patients are coma, lethargy, seizures and vomiting, unexplained developmental delay and siblings with similar symptoms. Plasma amino acids analysis is also ordered as a confirmational test to follow up abnormal newborn screening results. Hyperammonemia is characteristic to the most urea cycle disorders (**Table 3**) and therefore is another

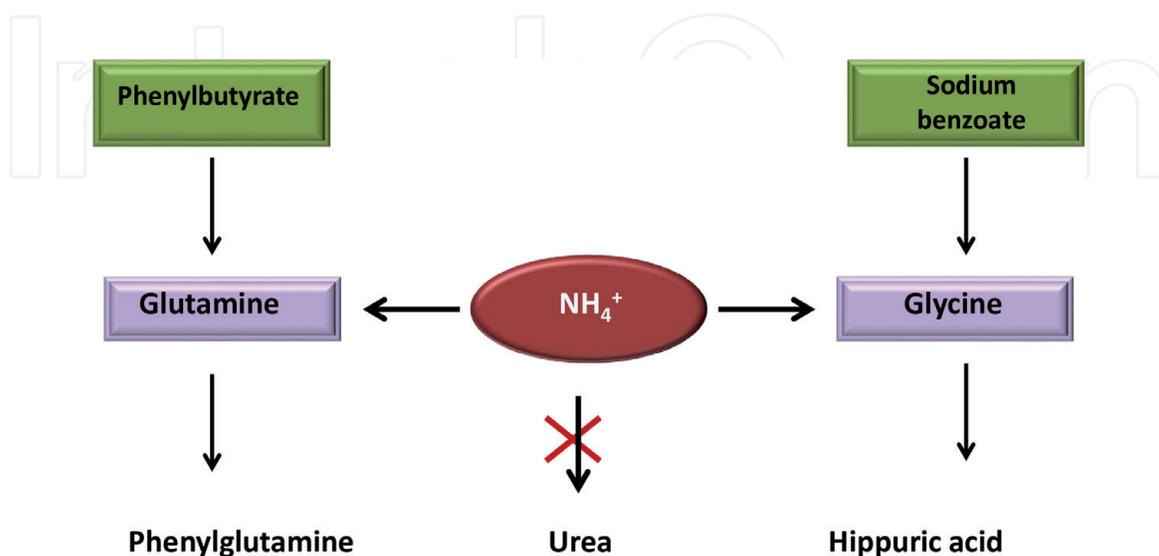


**Figure 11.**  
 Treatment strategies in amino acids disorders.

strong indication for plasma amino acids analysis. Additional general biochemical indicators of follow up quantitative amino acids analysis are ketosis (high blood and urine ketones), acidosis (blood pH below 7.35) and lactic acidemia (high lactate excretion), alkalosis (blood pH above 7.45), polyuria, polydipsia (extreme thirstiness), and dehydration. Amino acids analysis is also an important tool in the diagnosis of muscle and liver diseases, neurological disorders, renal failure, autism spectrum disorders and nutritional disturbances. Interpretation of amino acids profile is not just based on the abnormal level of a single amino acid, but rather involves pattern recognition, diagnostic ratios (Tables 1 and 2) and correlation to the patient's clinical history. It is recommended to confirm the diagnosis by molecular analysis or *in vitro* enzymatic assay (usually skin or tissue biopsy sample or blood cells).

## 5. Treatment options

Currently, there are numbers of available therapeutic approaches that aim in a substrate and downstream products restoration balance (Figure 11). One of the



**Figure 12.**  
 Removal of toxic ammonia. In urea cycle disorders ammonia cannot be converted to urea, but alternatively can be converted to glutamine and glycine. Ammonia scavengers phenylbutyrate and sodium benzoate react with glutamine and glycine and consequently remove excess of ammonia. Phenylglutamine and hippurate are excreted in urine.

approaches in this direction is to reduce substrate accumulation by dietary restrictions. Nutritional therapies restrict offending amino acid or often total protein consumption through provision and monitoring of all essential components to meet dietary requirements. For example, special medical foods for PKU affected individuals have a very negligible amount of phenylalanine, but supplement the total protein required for the normal growth, development and nutritional status. Another example is MSUD nutritional management that restricts intake of the branch chain amino acids [41], but supplies the majority of the protein required in the standard diet.

Amino acids disorders are often manifested by the accumulation of toxic downstream metabolites. For example, urea acid disorders are characterized by life-threatening hyperammonemia (ammonia accumulation). Toxic metabolites removal treatment aims at reducing production or increasing excretion of these metabolites. To reduce hyperammonemia, sodium benzoate and phenylbutyrate are used to increased ammonia excretion (**Figure 12**) and to bypass the urea cycle metabolic block [42, 43]. Another example is an approach to reduce the production of succinylacetone, a neurological toxin that accumulates in tyrosinaemia I. Nitisinone (NTBC) treatment blocks a formation of fumarylacetoacetate and its subsequent conversion to the succinylacetone [44, 45].

If as a result of mutation, a specific enzyme still retains its residual activity, it can be stimulated by a co-factor or a co-factor precursor supplementation. This concept applies in treating tetrahydrobiopterin deficiency (**Figure 2**) [46, 47], remethylation defects (**Figure 4**) [48] and cystathionine beta-synthase deficiency (**Figure 4**) [10]. In some amino acids disorders, even partial metabolic block prevents from synthesizing an essential downstream metabolite to meet metabolic requirements. In these cases, essential product supplementation is required. For example, as a part of urea cycle disorders management, L-arginine and L-citrulline are administered [43]. This helps to reduce excessive protein catabolism, due to the low arginine levels.

## 6. Conclusion

In conclusion, amino acids disorders are a group of inborn errors of metabolism with highly variable clinical and biochemical presentations. Clinical manifestation often comprises severe neurological symptoms, growth and developmental delays. Most of the amino acids disorders related conditions are included in the newborn screening program to facilitate early diagnosis and early disease treatment. The analysis of physiological amino acids levels is a key tool in the diagnosis and clinical management of inborn errors of amino acids metabolism. A small subset of amino acids is analyzed in newborn screening by tandem mass spectrometry and leads to the detection of affected neonates even when they do not have a symptomatic disease manifestation. A more comprehensive, quantitative amino acids analysis covers analysis of nearly 40 amino acids. Prior to the analysis and results interpretation, pre-analytical variables such as a fasting status and medication treatments should be taken into account in order to avoid false reported findings. A most common sample preparation method for the quantitative amino acids analysis is acidification of specimen with a known small volume of concentrated acid, such as sulfosalicylic acid to precipitate proteins and large molecules, followed by centrifugation, leaving the water-soluble amino acids in the supernatant for the analysis. A variety of analytical methods have been developed over the past 60 years, and scientists have made significant achievements in the fields of derivatization, chromatography and mass spectrometry, however, the ion exchange chromatography method still

remains the gold standard technique in the field. It is expected that more advanced techniques will be developed targeting important clinical laboratories requirements such as reduced samples pretreatment, linearity over the large concentration range for over the 40 amino acids, increased automation, high sensitivity, shorter run time and improved specificity. These methodological improvements will facilitate the diagnostic process and therapy monitoring for amino acids disorders. The field is also expanding to the more exploratory platforms such as a whole-genome sequencing and untargeted metabolomics. Although these modalities have some restrictions in clinical settings [1, 49], they facilitate novel genes identification, novel biomarkers discovery and disease associations and thus strongly advancing the field [50, 51].

The major treatment goal for amino acids disorders is to normalize imbalance between the substrate and its downstream products and to avoid accumulation of the toxic substances. At the same time, nutritional management must meet basic dietary requirements for growth and normal development. Even though for many amino acids disorders current treatments do not offer a cure, they significantly improve the quality of life. It is expected over the upcoming years, that methodological advances will lead to a greater understanding of the IEM and in particular amino acids related disorders which will help further to improve disease outcomes.

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## Author details

Yana Sandlers  
Cleveland State University, Cleveland, OH, USA

\*Address all correspondence to: [y.sandlers@csuohio.edu](mailto:y.sandlers@csuohio.edu)

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