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Originally published:

December 2016

Journal of Biotechnology 242(2017), 30-54

DOI: https://doi.org/10.1016/j.jbiotec.2016.12.001

Perma-Link to Publication Repository of HZDR:

https://www.hzdr.de/publications/Publ-24465

The Why and How of Amino Acid Analytics in Cancer Diagnostics and Therapy

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Acknowledgements: Our work related to amino acid detection and metabolic targeting has been supported by the European Social Fund (ESF) and Free State of Saxony, the Else Kröner-Fresenius Foundation and the German Academic Exchange Service (DAAD). OncoRay is funded by the BMBF in the program “Center for Innovation Competence“. We thank Mrs. Melanie Hüther and Mr. Jürgen Löbner for their efforts and technical assistance as well as Mrs. Christiane Kunz B.A. for excellent design advice.

Conflict of Interest: The authors claim no conflict of interest.

Abbreviations

AA - amino acid/amino acids; AAD – amino acid detection; ADI – arginine deiminase; ADT – arginine deprivation therapy; ALL – acute lymphoblastic leucemia; APCI - atmospheric pressure chemical ionization; APPI - atmospheric pressure photo ionization; AQC - aminoquinolyl-N-hydroxysuccinimidyl carbonate (AQC); CE - capillary electrophoresis; CI - chemical ionization; dabsyl-Cl – 4-(4-dimethylaminophenylazo)benzene sulfonyl chloride; dansyl-Cl – 5-(dimethylamino)naphthalene-1-sulfonyl chloride; EFSA - European Food Safety Authority; EI - electron impact ionization; ESI - electrospray ionization; Fmoc-Cl - fluorenylmethyloxycarbonyl chloride; GBM – glioblastoma multiforme; GC - gas chromatography; HCA – heterocyclic amines; HILIC - hydrophilic interaction liquid chromatography; HPLC - high performance liquid chromatography; LC-MS - liquid chromatography mass spectrometry; LLOQ - lower limit of quantification; LOD - limit of detection; MR - maillard reaction; MRM - multiple reaction monitoring; MS - mass spectrometry; MS/MTR - methionine synthase/5-methyltetrahydrofolate-homocysteine methyltransferase; MTAP - methylthioadenosine phosphorylase; OPA - *o*-phthalaldehyde; PET – positron emission tomography; PITC - phenylisothiocyanate; PKU - phenylketonuria; RP - reversed phase; RPLC – reversed phase high performance liquid chromatography; SPE - solid phase extraction; TFA – trifluoroacetic acid; UHPLC - ultra high performance liquid chromatography; VOCs – volatile organic compounds

Abstract [1]

Pathological alterations in cell functions are frequently accompanied by metabolic reprogramming including modifications in amino acid metabolism. Amino acid detection is thus integral to the diagnosis of many hereditary metabolic diseases. The development of malignant diseases as metabolic disorders comes along with a complex dysregulation of genetic and epigenetic factors affecting metabolic enzymes. Cancer cells might transiently or permanently become auxotrophic for non-essential or semi-essential amino acids such as asparagine or arginine. Also, transformed cells are often more susceptible to local shortage of essential amino acids such as methionine than normal tissues. This offers new points of attacking unique metabolic features in cancer cells. To better understand these processes, highly sensitive methods for amino acid detection and quantification are required. Our review summarizes the main methodologies for amino acid detection with a particular focus on applications in biomedicine and cancer, provides a historical overview of the methodological pre-requisites in amino acid analytics. We compare classical and modern approaches such as the combination of gas chromatography and liquid chromatography with mass spectrometry (GC-MS/LC-MS). The latter is increasingly applied in clinical routine. We therefore illustrate an LC-MS workflow for analyzing arginine and methionine as well as their precursors and analogs in biological material. Pitfalls during protocol development are discussed, but LC-MS emerges as a reliable and sensitive tool for the detection of amino acids in biological matrices. Quantification is challenging, but of particular interest in cancer research as targeting arginine and methionine turnover in cancer cells represent novel treatment strategies.

[1] This comprehensive review aims at presenting and discussing the amino acid detection tools of interest in biomedical science and cancer research but does not claim to cover the entire field and literature, respectively. The authors apologize to those researchers whose work has not been mentioned due to restrictions in space and time.

1. From Food Chemistry to Biomedicine: Why to detect AAs

1.1 Background

Amino acids (AAs) – both containing amine (-NH2) or carboxylic acid (COOH) functional groups – were already described in the early 19th century commenced with the isolation of asparagine from *Asparagus sativus* (Vauquelin and Robiquet, 1806). It took another six decades to elucidate the structure of asparagine (Kolbe, 1862). Today, the presence and structure of more than 300 AAs are known (Wu, 2009) including the 21 proteinogenic AAs (selenocysteine and the 20 canonical AAs) which are required for protein biosynthesis in living organisms (Gao et al., 2015). Free and protein/peptide-bound AAs occur ubiquitously in the biosphere, in microorganisms, plants and animals, and are thus an integral nutritional component in species-appropriate food. AAs are specified as essential, semi-essential or non-essential depending on their actual metabolic availability within the (human) body and the divergent necessity for exogenous supply. All AAs except glycine have an asymmetric (α-) carbon with carboxylic or amino groups and are optically active; their nonpolar, polar and unloaded or loaded side chain is crucial for chemical verification and defines the AA classification.

Due to the omnipresence as well as the high variability of AA pattern in various matrices, AA detection (AAD) has become an integral part in life science. The quantification of AAs is primarily based on chromatographic technologies. Attributes important for chromatographic detection are the ampholytic character of AAs leading to a zwitter ion at the individual isoelectric point (IP), as well as the exclusive presence of sulfur in methionine and cysteine, which - amongst others - defines three-dimensional protein folding via the formation of disulfide bonds (Chaimbault et al., 1999; Wu, 2009). Methods of choice for AAD are high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) (Krumpochova et al., 2015; Otter, 2012; Poinsot et al., 2010; Rigas, 2013), all of which can nowadays be combined with mass spectrometry (MS) to increase the sensitivity of detection as will be highlighted in more detail later in this essay.

The –omics era of the 21st century is unconceivable without sensitive and selective state-of-the-art AA analytics, in particular with the expansion from genomics to proteomics and most recently metabolomics approaches (Arapitsas et al., 2016; Becker et al., 2012; Jehmlich et al., 2015; Possemato et al., 2011; Prabhu et al., 2014). However, the field of applications for both classical as well as modern AA detection techniques is diverse as outlined in Fig. 1. It ranges from biological disciplines including zoology (Preston, 1993; Wu et al., 2016) marine research (Bermudez et al., 2015) and anthropology (Kaal et al., 2016), where samples as diverse as sediments (Larsen et al., 2015), tissues (Wu et al., 2016) and archeological materials (age determination) are monitored, to the use in biotechnology and pharmaceutical research and industry, e.g. for quality control and drug design (Holzgrabe et al., 2010; Ilisz et

al., 2006; Peura et al., 2013; Vlaardingerbroek et al., 2013). AAD can be performed in diverse liquid and solid matrices including geological samples, bacteria and plant masses, DNA/protein or RNA/protein extracts, blood, serum and plasma, urine and stool as well as tissue and biopsy samples. Indeed, body fluids are of particular interest in biomedicine and clinical chemistry.

1.2 Linking Food Chemistry and Public Health

Food chemistry is a major area of application for AAD. The AA composition of particular food products is for example analyzed to calculate a protein digestibility-corrected AA score (PDCAAS) as measure for nutritional protein quality based on the amino acid profile and the human requirements for essential AAs (Bellomaria et al., 2016; Rutherfurd et al., 2015; Sarwar, 1984; Schaafsma, 2000). At the same time, the AA composition and abundance may affect food quality and thus needs to be monitored already during production. As an example, free AA and ammonium are the main nitrogen source for alcoholic fermentation by yeast. Reduced AA concentrations can lead to malfermentation whereas the excess of AAs might result in microbiological instability, both counterproductive for high quality wine production. Here, AAs as well as specific noxious derivatives, such as biogenic amines that serve as an indication for microbiological deterioration, are routinely determined via HPLC and CE (Acunha et al., 2016; Ortega-Heras et al., 2014; Wang et al., 2014). Biogenic amines are decarboxylated AAs and nitrogenous organic compounds that can act as neurotransmitters and precursors for hormones and vitamins (Karovicova and Kohajdova, 2005; Rai et al., 2013). Their accumulation in the body has been linked to several adverse health effects such as headache and allergic reactions (Binner et al., 2013; Rabie et al., 2014; Santos 1996). For this reason, the European Food Safety Authority (EFSA) has proposed a risk assessment for fermented foods based primarily on the most abundant biogenic amines histamine and tyramine (EFSA, 2011).

Another interface between AA derivatives, food chemistry and public health is the presence of Amadori compounds in various food products which serves as an indicator for the extent of heating, e.g. during milk processing via ultra-high temperature treatment and conventional sterilization (Mehta and Deeth, 2016; Wellner et al., 2009). The direct detection of these glycated AAs is in principle adapted from AAD technologies (Hellwig and Henle, 2014; Lee et al., 2015). Amadori compounds are bright and unscented products of the first step of the maillard reaction (MR) which is a complex biochemical reaction between free AAs or proteins and carbohydrates (Galli, 2007) also called “non-enzymatic browning” due to the formation of aromatic, mostly colored end stage reaction products during cooking in the absence of enzymes. Indeed, both beneficial and hazardous MR products may be formed depending on the way of food processing as emphasized in a recent review by Tamanna and Mahmood (2015). Major issues in agriculture and food preparation are thus the enhancement and

preservation of nutritional value on the one hand and the avoidance of harmful ingredients such as heterocyclic amines (HCAs) and acrylamide on the other hand. The latter is mainly produced in an amylaceous environment via biochemical modification of asparagine (Lineback et al., 2012; Yaylayan et al., 2005) and was already classified as human carcinogen (Group 2A) by the International Agency for Research on Cancer in the World Health Organization (IARC-WHO) (IARC, 1994) in 1994. Still, there are no standard regulations on HCAs, although roughly at the same time the IARC had appointed several different HCA species with carcinogenic potential (IARC, 1993; Lee et al., 2015; Tamanna and Mahmood, 2015).

Human condition is certainly influenced by the daily ingested AA content and composition. This becomes evident in particular in patients with diseases related to AA incompatibilities and enzyme defects (van Vliet et al., 2014). Fast and reliable detection of AA misbalances in blood, plasma, serum, urine and liquor samples is thus of tremendous importance and routine in clinical chemistry. Classical clinical indications for AA analysis are neurological symptoms or dysregulations, metabolic imbalances and acidosis, gastrointestinal disorders, kidney diseases and sepsis as well as the monitoring of patients in the course of a diet (Batch et al., 2014; Fitian et al., 2014; Niewczas et al., 2014). Dietetic food with particular nutritional compositions is commercially available for selected groups of people not only for energy restriction during weight loss but in particular for infants and young children and for people with gluten intolerance or other specific medical conditions as depicted. The application of AA analytics for quality control of such special food for infants and medical purposes is now directed for all EU countries via EU-Regulation No 609/2013.

1.3 Amino acid-associated diseases

The detection of AA-associated diseases is especially important in newborns. The newborn screening which is standard in many well-developed countries was established to identify apparently healthy infants with serious inherited, monogenic disorders early enough so that they can be treated by drug or dietary interventions before exhibiting clinical symptoms (Pourfarzam and Zadhoush, 2013; Summar et al., 2013; Therrell et al., 2014). Indeed, a range of inborn errors of metabolism (IEM) with high morbidity or mortality if left untreated can be identified by a single test via an abnormal AA profile in a blood spot of the neonate (Lehotay et al., 2011; Therrell et al., 2014). In most cases, the analysis is performed via liquid chromatography combined with mass spectrometry (LC-MS) (Becker et al., 2012; la Marca, 2014; Lehotay et al., 2011; Pitt, 2009; Zurawicz and Kaluzna-Czaplinska, 2015).

Each specific disorder detected via the newborn screening is rare, but their cumulative incidence accounts to 1:1,500 – 1:5,000 live births calling for a worldwide action (Pourfarzam and Zadhoush, 2013; Zhang et al., 2000). Hereditary phenylketonuria (PKU) was the first

disease tested with a screening method in the 1960's and is part of the newborn screening with high relevance. PKU leads to an accumulation of phenylketons in the urine without synthesis of tyrosine; the ratio of respective AAs in the blood is indicative for diagnosis (Shushan, 2010; Staudigl et al., 2011). PKU is asymptomatic in newborn, but untreated it leads to irreversible cognitive impairment, hyperactivity, autistic-like behaviors and seizures. The therapy consists of a lifelong dietary phenylalanine restriction combined with tyrosine supplementation to control the disease (Al Hafid and Christodoulou, 2015; Blau et al., 2010; Therrell et al., 2014). Other AA disorders with similar health effects include urea cycle malfunctions leading to citrullinemia or argininaemia and resulting in hyperaminoacidurias (van Vliet et al., 2014). Here, the dietetic treatment comprises protein restriction and food supplementation with arginine. Despite these well-described metabolic disorders, there is increasing evidence that other developmental damage manifestations are reflected by and causally relate to AA misbalances. As an example, Evans et al (Evans et al., 2008) systematically analyzed the plasma AA profile in 34 autistic children using HPLC and GC and concluded from their data that autism may be attributed to an imbalance of neurotransmitters caused by the abnormal AA pattern in the blood. Today, the respective AA status is discussed as a biomarker profile for the early diagnosis of autism (Evans et al., 2008; Naushad et al., 2013; Zurawicz and Kaluzna-Czaplinska, 2015). In gerontology, the relevance of the AA-neurotransmitter axis was in principle recognized in the context of dementia and Alzheimer's disease already in the 1980s (Degrell et al., 1989; Tarbit et al., 1980). However, it has come into focus again only recently, when specific AAs such as arginine, methionine, glutamine/glutamate and/or asparagine/aspartate were shown to be altered in the plasma, cerebrospinal fluid and/or brain tissue of patients (Gueli and Taibi, 2013) as well as in mouse and rat models of Alzheimer's disease (Kan et al., 2015; Xing et al., 2016).

AAD is of course highly informative in all age groups including juveniles and young adults. While glycated hemoglobin A1c (HbA1c) is an approved general biomarker in patients with diabetes (Jones et al., 2014; Nathan et al., 2014), aromatic and branched chain AAs are particularly associated with insulin resistance in Type 2 diabetes (Giesbertz and Daniel, 2016; Morris et al., 2012; Stancáková et al., 2012; Wiklund et al., 2014). Indeed, several recent studies each based on cohorts of >1,500 individuals (up to 5,000) from different selected populations including Europe demonstrate the predictive value of these specific AAs for risk assessment in normoglycemic adults to develop diabetes, metabolic syndrome and/or cardiovascular disease (Magnusson et al., 2013; Tillin et al., 2015; Wuertz et al., 2013; Yamakado et al., 2015).

The examples given above reveal that AAs play an important role in numerous metabolic disorders. Malignancies are based on genetic alterations but may also be considered as

metabolic diseases. Aerobic glycolysis (Warburg effect) and the development of spatiotemporal regions of hypoxia and tissue acidosis are the best known and well-studied phenomena in this context (Cairns et al., 2011; Jang et al., 2013) but not exclusive. Indeed, cell metabolism is reprogrammed during carcinogenesis through complex genetic and epigentic dysregulation of a variety of metabolic enzymes in glycolysis, tricarboxylic acid cycle, oxidative phosphorylation and even beyond including alterations in AA metabolism (Ghaffari et al., 2015; Li et al., 2016). This on the one hand may induce alternative crosstalk or feedback mechanisms and restore energetic plasticity to support tumor cell survival and proliferative activity, but on the other hand also opens a window for therapeutic intervention. The relevance of AA supply and the potential for AA metabolic targeting in cancer shall thus be particularly highlighted in the next chapter as it requires state-of-the-art AAD technology.

2. AA quantification: Increasing demand in cancer research and treatment

2.1 The challenge of tumor metabolism

More than 14 million new cases of cancer and >8 million cancer-related deaths have been reported worldwide in 2012/2013 with an ongoing upward trend – these are the alarming numbers derived from the GLOBOCAN study directed by the WHO International Agency for Research on Cancer (IARC)(Ferlay et al., 2015) and the GBE initiative at the Institute for Health Metrics and Evaluation (IHME) at the University of Washington (Fitzmaurice et al., 2015). Malignancies are thus among the top non-communicable lethal diseases globally, and in particular in the well-developed countries with their aging population. Remarkable progress in surgical techniques, in image-guided photon and particle therapy as well as in the development of numerous therapeutic treatment options - ranging from classical and novel chemotherapeutic drugs to a broad range of individually applicable targeted therapeutics - have led to a significant improvement in the clinical management of some cancer types. However, the overall cure rate has slightly increased with some malignant diseases remaining an unsolved therapeutic mystery such as pancreatic cancers and glioblastomas, thereby underlining the continuing demand for innovative strategies in diagnostics and therapy (Stewart and Wild, 2014).

Several researchers have shown that body fluids of healthy controls and breast cancer patients vary significantly in the levels of some AAs (Barnes et al., 2014; Cascino et al., 1995; Cheng et al., 2015; Poschke et al., 2013). The same was reported for lung (Cascino et al., 1995; Cobo Dols et al., 2006; Shingyoji et al., 2013; Zhao et al., 2014), ovarian (Maruyama et al., 2014), head and neck (Cobo Dols et al., 2006), gastric (Fan et al., 2012; Miyagi et al., 2011) and pancreatic (Fukutake et al., 2015; Miyagi et al., 2011) as well as colorectal cancer patients (Bener et al., 2006; Leichtle et al., 2012; Yatabe et al., 2013).

These findings imply that AA profiling in plasma/serum, other body fluid or selected tissue samples might be a new tool for early diagnosis of various cancers as suggested by some authors and also summarized in (Simińska and Koba, 2016). At least, AA analysis can complement other innovative strategies for early disease detection such as the monitoring of volatile organic compounds (VOCs). Volatolomics approaches have for example been applied for the profiling of physicochemical changes in both invasive and non-invasive cancers (Le et al., 2014; Vishinkin and Haick, 2015; Broza et al., 2015) and are useful as diagnostic tools for several cancer entities, e.g. in prostate (Khalid, 2015}, breast (Barash et al., 2015) and lung (Gasparri et al., 2016). Depending on the analyte(s) of interest, volatolomics can be performed by array-based biosensing techniques with recognition elements and suitable transducers (Gasparri et al., 2016; Le et al., 2014) or via GC-MS by analyzing the headspace of a sample (Barash et al., 2015; Khalid et al., 2015). However, AAs are non-volatile and cannot be directly measured by such approaches as they require derivatization as detailed in chapters 3 and 4. Nonetheless, AA profiling might be diagnostically informative, but by itself may not be adequate for the design of novel metabolic targeting strategies. Indeed, detailed mechanistic insight into cancer-specific metabolic alterations including AA anabolism and catabolism is necessary for the design of well-directed metabolic interventions.

After decades of research to mechanistically understand the Warburg effect phenomenon and to unravel the complex face of the tumor metabolome beyond aerobic glycolysis and therapeutically-relevant hypoxia-driven adaptations (Hirschhaeuser et al., 2011; Horsman and Vaupel, 2016; Jose et al., 2011; Marchiq and Pouyssegur, 2016; Tatum et al., 2006), metabolic targeting and (anti)-metabolic therapy, respectively, are still in their infancy. One of the reasons for early disappointment and delayed progress in this field could be that a benefit for patient outcome might manifest primarily or exclusively after combined treatment, e.g. via multi-metabolic targeting and/or in combination with curative approaches such as radiotherapy. Energy metabolism has gained particular attention because of the cancer cells' exorbitant energy demand due to high proliferation rates and an abnormal micromilieu (Ghaffari et al., 2015). Indeed, energy resources are not only required for lipid and macromolecule production during proliferation but also for DNA synthesis and repair (Dang, 2012; Scanlon and Glazer, 2015). However, several intermediate metabolic programs are also altered in (proliferating) tumor cells including the turnover of proteinogenic amino acids which is closely linked to the mTOR and ER stress response pathways and offers additional points of attack for metabolic targeting. A comprehensive overview on recent findings related to the regulation and crosstalk of glucose, fatty acid and amino acid metabolic pathways highlighting some relevant cancer therapy strategies is given in (Li and Zhang, 2016).

In general, cancer cells appear to very efficiently utilize all physiological exogenous sources to cover their enhanced demand for proteinogenic AAs and even the absence of non-essential AAs can disturb the delicate (abnormal) balance in tumor metabolism. Evidentially, they might transiently or permanently become auxotrophic for non-essential or semi-essential amino acids and are also often more susceptible to local shortage of essential amino acids than normal tissues. In the following paragraphs, we will therefore exemplify the rational and challenge for designing systemic dietary or enzymotherapeutic amino acid deprivation therapies on the basis of each one non-essential, one semi-essential and one essential AA (asparagine - arginine – methionine; Fig. 2) (Agrawal et al., 2012; Bertino et al., 2011; Tsai et al., 2016). It is noted though, that uptake of various, even non-essential proteinogenic AAs correlates with cancer cell proliferation, and that reduced exogenous supply can critically affect proliferative activity, survival or tumorigenic potential of malignant cells. Amongst others, these include glutamine (Jain et al., 2012; Mullen et al., 2012) which is not only required in protein synthesis but can enter the citric acid cycle via the α-ketoglutarate intermediate triggering aerobic glutaminolysis (Fig. 3), as well as serine which is interconnected with glycine, involved in nucleotide de novo synthesis or may undergo serinolysis to enter the glycolytic pathway playing a role in aerobic glycolysis of cancer cells (Labuschagne et al., 2014; Maddocks et al., 2013 and 2016; Possemato et al., 2011). Serine is also linked to the methionine pathway by providing ATP and one-carbon units to regenerate methionine from homocysteine thereby indirectly affecting the transfer of methyl groups to DNA and RNA (Maddocks et al., 2013 and 2016). These two examples indicate that different regulatory pathways contribute to the cancer cells' sensitivity to AA depletion but do not exclusively relate to stress responses commonly seen under nutrient deficiency - and in this case transmitted by an insufficient protein formation and potential misfolding.

Amino acid auxotrophy describes the phenomenon of malignant cells to develop full dependency on the exogenous supply of selected single AAs that are *per se* not essential for normal cells and tissues (Agrawal et al., 2012; Bach and Swaine, 1965). This is basically due to an insufficient enzyme apparatus for intracellular *de novo* synthesis of these AAs, in some cancers caused by genetic or epigenetic alterations. In principle, carcinomas are affected to the same degree as sarcomas, melanomas or lymphomas (Bowles et al., 2008; Kim et al., 2009; Savaraj et al., 2015; Tomlinson et al., 2015). However, the clinically most relevant example still is the dependency of acute lymphoblastic leukemia (ALL) cells on extracellular asparagine which was discovered five decades ago (Broome, 1963; Haley et al., 1961; Horowitz et al., 1968). Subsequently, systemic treatment with the asparagine-degrading enzyme asparaginase was introduced into clinical practice already in the 1970s (Dores et al., 2012; Pieters et al., 2011; Rytting et al., 2014).

2.2 Asparagine targeting: An example of clinical success in ALL

Today, asparagine depletion by treatment with bacterial-derived type II L-asparaginases which degrade asparagine (Fig. 2) to aspartate and ammonia is an integral component of pediatric ALL therapy and has improved survival from < 5% to more than 80% (Schrappe et al., 2012). The enzymotherapy is also applied in the treatment of Non-Hodgkin lymphomas in combination with various other therapeutic agents (d'Amore et al., 2015; Dreyling et al., 2013; Wang et al., 2015). Of note, type II asparaginases are induced in bacteria under aerobic conditions only and have a highly specific (but not exclusive) asparagine activity while type I arginases are expressed independently of the environmental condition and similarly hydrolyze L-asparagine and L-glutamine (Batool et al., 2016). The main drawbacks of enzymotherapy with L-asparaginase are symptomatic or asymptomatic allergic (hypersensitivity) reactions which are accompanied by loss of enzyme activity.

Essential progress in biochemical and bioengineering practice in the last century allowed the production of the first PEGylated form of asparaginase, aspargase, with superior characteristics (Abuchowski et al., 1979). In general, enzyme PEGylation aims at improving pharmacokinetics and pharmacodynamics by prolonging the circulation half-life of the therapeutic enzyme in the human body and increasing its stability under physiological conditions; in addition, PEGylated enzymes might be less immunogenic (Harris and Chess, 2003; Jiang et al., 2003; Roberts et al., 2012; Zhang et al., 2014). By now, three different forms of L-asparaginase have successfully been developed into commercial drugs, i.e. naïve *E.coli* asparaginase, PEGasparaginase and an asparaginase derived from the bacterium *Erwinia chrysanthemi* (Ali et al., 2016; Avramis et al., 2007; Pieters et al., 2011). These can be given in first, second and third line therapy due to their different immunogenic profile (Tong et al., 2013). The application, proposed mode of action, side effects and limitations of these therapeutic enzymes in childhood ALL have been summarized in numerous review articles and consensus reports published over the past five years (Ali et al., 2016; Asselin and Rizzari, 2015; Batool et al., 2016; Hijiya and van der Sluis, 2016; Pieters et al., 2011; Tong et al., 2013; van der Sluis et al., 2016). The reference list provided here is not exhaustive but highly informative and includes some articles which comprehensively describe and recapitulate the various alternative sources for L-asparaginase production ranging from numerous, mainly gram-negative bacteria via fungi and yeast to algae and actinomycetes which may well become clinically relevant in the future.

The benefit of asparaginase therapy in particular for pediatric ALL patients is beyond dispute. However, striking recent observations indicate that the underlying mechanism of asparaginase sensitivity in ALL is still not fully understood. Based on an early study in a limited number of ALL patients (Haskell et al., 1969) followed mainly by cell line and animal experiments (Aslanian et al., 2001; Hutson et al., 1997; Peng et al., 2001), it was thought for

a long time that asparagine auxotrophy in ALL is exclusively due to low baseline or lack of expression of the enzyme arginine synthetase (ASNS), and that resistance to asparaginase treatment relates to an adverse (up)regulation of ASNS expression and/or activity upon treatment. However, the mechanistic interrelation began to totter when Dübbers (2000) documented that ASNS activity in acute lymphoblasic (ALL) and acute myeloblastic (AML) cell preparations from patients were highly variable and did not significantly differ. Differences in ASNS activity were only seen when they considered distinct subgroups of leukemia, i.e. AML-M5 (acute monocytic leukemia) and B-lineage ALL blast cells showed lowest ASNS activities (Dübbers et al., 2000). Stams and coworkers (Stams et al., 2003) later found no correlation between ASNS expression and sensitivity to L-asparaginase in a specific subgroup of pediatric ALL which is per se known for its high cellular sensitivity to the treatment and better clinical outcome (Ramakers-van Woerden et al., 2000); the subgroup accounts to about 25% of all ALL and is characterized by a (12;21) chromosome translocation carrying a *TEL/AML1* fusion gene. The observations were later confirmed in several other ALL (sub-)groups (Appel et al., 2006; Hermanova et al., 2012; Richards and Kilberg, 2006; Su et al., 2008).

Comprehensive gene expression profiles in numerous ALL cell lines and patient materials were recorded by Fine and colleagues (2005). Overall, a consistent pattern of gene expression changes in cell lines and clinical samples could be identified upon *in vitro* asparagine starvation. This was independent of genetic background and chromosomal translocations in the ALL models, and did not reflect response to treatment (Fine et al., 2005). Nonetheless, the data revealed an upregulation of ASNS upon enzymotherapeutic asparagine withdrawal in asparaginase-resistant but not –sensitive cell lines, whereas neither baseline ASNS expression nor its change after treatment *in vitro* were predictive for L-asparaginase susceptibility of clinical samples. Indeed, a response classifier gene profile defined from the cell line data including ASNS failed in the clinical sample cohort suggesting that mechanisms of resistance other than ASNS gene and protein regulation are relevant. Different hypotheses are under investigation ranging from ASNS polymorphisms associated with additional or alternative functions of the resulting protein (Akagi et al., 2009; Ben Tanfous et al., 2015; Pastorczak et al., 2014) to the adverse impact of ALL supportive, ASNS highly expressing bone marrow–derived stroma cells (Iwamoto et al., 2007). In addition, the well-known adverse effect of silent inactivation due to patient-specific immune reactions and antibody production against the enzyme or particular drug compartments such as PEG has to be considered in this context (Ali et al., 2016; Asselin and Rizzari, 2015; Pieters et al., 2011; Tong et al., 2014; van der Sluis et al., 2016). These findings are also relevant for expanding the treatment to other cancers as those listed in Fig. 4.

The direct detection of asparagine levels in patient serum for enzymotherapy monitoring seems to be straight forward but turned out to be quite delicate. In a recent consensus report related to L-asparagine treatment in pediatric ALL patients, van der Sluis (van der Sluis et al., 2016) critically discuss this option concluding that it is impractical and unreliable for clinical use due to the rapid *depletion* of asparagine *ex vivo* in presence of asparaginase in patient blood samples. Furthermore, data interpretation appeared to be difficult due to the non-standardized definition of cut-off values for complete asparagine depletion. As an alternative, the assessment of asparaginase activity in blood/plasma samples turned out as a reproducible and reliable tool for treatment evaluation in clinical routine (van der Sluis et al., 2016). This is meaningful because serum asparagine levels significantly correlated with *E. coli*-derived asparaginase serum activities in several earlier studies (Avramis et al., 2007; Douer et al., 2007; Pieters et al., 2008). Measuring of asparagine levels was thus required to approve asparaginase activity as readout and is still used in experimental settings where researchers can more easily comply with rapid, demanding sampling procedures. Anyways, the detection of amino acid level in pharmacodynamics and metabolic targeting studies seems more common for monitoring arginine withdrawal strategies which shall be highlighted next (Ensor et al., 2002; Izzo et al., 2004; Kelly et al., 2012; Mauldin et al., 2012; Stone et al., 2012a).

2.3 The potential of arginine deprivation therapy

The addiction of tumor cells to exogenous arginine was recognized already in the last century when mycoplasma contaminations afflicted many labs worldwide manifested in growth arrest in animal cell cultures. This was mainly due to the fact that some mycoplasma strains utilize enzymatically degraded arginine for energy production leading to an arginine deficiency in the contaminated cell cultures (Altucci et al., 1966; Capiaumont et al., 1995; Fenske and Kenny, 1976). Systematical depletion of arginine for cancer treatment, i.e. arginine deprivation therapy (ADT), has been developed more recently as a new promising enzymotherapeutic anti-cancer strategy which has proven anti-proliferative and/or pro-apoptotic effects in various cancer cells *in vitro* and *in vivo* (for review see Fultang et al., 2016 and Qiu et al., 2015) (Fig. 4). Besides protein biosynthesis, arginine (Fig. 2) as a nitrogen-rich semi-essential amino acid is for example vital for the production of NO, creatine, and urea, and also contributes to intracellular proline and glutamate synthesis. Under certain physiological and pathophysiological conditions, such as growth or stress, arginine can be *de novo* synthesized intracellularly from ornithine (mainly in liver and kidney) or citrulline (basically in all mammalian cell types) via selected steps and enzymes of the urea cycle as illustrated in Fig. 3 (Morris, 2006; Shambaugh, 1977). The citrulline level in human plasma is lower than arginine by about a factor of two and may not be sufficient for complete compensation. However, citrulline availability is most probably not limited due to a constant

uptake from the gut as well as production in the mitochondria of enterocytes from glutamine and proline via the three key regulatory enzymes Pyrroline-5-carboxylate synthase, N-acetylglutamate synthase, and proline oxidase. The regulatory processes determining the citrulline resources via the gut are quite complex, but high arginine levels in the blood were shown to reduce this channel of supply (Breuillard et al., 2015; Curis et al., 2007; van de Poll et al., 2007; Fujita and Yanaga, 2007; Wu, 2009).

The enzymes converting arginine from ornithine and/or citrulline are expressed in a cell type, functional and differentiation-associated manner (Husson et al., 2003). Their absence or downregulation can cause auxotrophy for arginine. Reduction or lack of ornithine transcarbamylase (OTC), which is primarily expressed in normal hepatocytes, is frequently seen in auxotrophic hepatocellular carcinomas (Feun et al., 2008; Sugimura et al., 1992). In other organs, modifications in argininosuccinate synthetase (ASS-1) rather than argininosuccinate lyase (ASL) seem to play a major role in the development of cancer cell auxotrophy such as melanoma, small cell lung cancer, pancreatic cancers and others (Delage et al., 2012; Dillon et al., 2004; Hu and Cheung, 2009; Khoury et al., 2015; Qiu et al., 2014).

Several studies imply that the expression of ASS-1 is epigenetically down-regulated in arginine auxotrophic tumors via methylation of the ASS-1 promoter in the CpG island region (Delage et al., 2012; Dillon et al., 2004). In normal cells, lack of arginine leads to cell cycle arrest - if cells were not yet in G_0 phase and/or differentiated, and, most importantly, ASS protein levels increase to enhance the citrulline-to-arginine conversion for survival and maintenance of cellular AA homeostasis. Cancer cells may not only have lost this regulatory mechanism, but they also seem to be highly susceptible to arginine deficiency because of their abnormal cell cycle regulation. The latter is supported by the strong combinatorial effects of ADT demonstrated even in some ASS-positive malignant cells (Bobak et al., 2016; Vynnytska-Myronovska et al., 2013 and 2016; Kurlishchuk, 2016). In the absence of arginine, cancer cells also stop growing. However, subsequent cell cycle arrest might be poorly controlled so that some of them cannot maintain homeostasis, undergo autophagy and/or directly activate signaling pathways to apoptosis similar to the responses seen in cancer cells lacking ASS (Bobak et al., 2016; Delage et al., 2012; Gong et al., 2000; Pardee, 1974; Qiu et al., 2015; Shuvayeva et al., 2014).

The reduction of blood and tissue as well as cell culture arginine level can be achieved by enzymotherapy. While cell culture studies can also be performed with arginine-free media compositions, arginine-free diets are insufficient for critically reducing arginine-levels in the body. There are mainly two enzymes that can be applied for this purpose: arginine deiminase (ADI), e.g. from *Mycoplasma arginini,* which hydrolyses arginine into citrulline and ammonia, and recombinant arginase which degrades arginine to ornithine and urea. The xenobiotic ADI

shows pharmacodynamics advantages which made it beforehand more attractive to clinicians and is thus better investigated (Patil et al., 2016). The enzymes are usually PEGylated for *in vivo* use (Bobak et al., 2016; Miyazaki et al., 1990; Savaraj et al., 2010). ADI is suitable for ASS negative tumors due to the conversion into citrulline which could reduce efficacy in ASS positive cells; ASS expression was therefore suggested as biomarker for ADI sensitivity (Han et al., 2016; Kelly et al., 2012). Today, PEGylated ADI is commercially available and applied in several clinical phase I/II and III studies for patients with advanced hepatocellular carcinoma (NCT 01287585), small cell lung cancer (NCT 01266018) and breast cancer (NCT 01948843). It is also tested in combination with chemotherapeutics such as Gemcitabine and Paclitaxel for the treatment of patients with pancreatic carcinoma (NCT 02101580).

Arginase as an enzyme of the urea cycle is less immunogenic and exists in two isoforms: Arginase I is expressed in the liver while arginase II can be found in the mitochondrial matrix of roughly all extrahepatic cells, in particular in the kidney (extensively reviewed by Sidney M. Morris jr. and colleagues (Morris, 2002; Morris 2006; Morris, 2009; Wu and Morris 1998)). A PEGylated recombinant arginase I is preferentially used for enzymotherapy. The enzyme was improved during the past decade by modern biochemical engineering approaches. Indeed, its affinity to arginine as well as the rather moderate catalytic activity under physiologic conditions could be essentially enhanced by replacing Mn^{2+} with Co^{2+} in the active center (Stone et al., 2010 and 2012a). Recombinant arginase I is now also in clinical trials for hepatocellular carcinoma (NCT 00988195), renal cell carcinoma, melanoma and prostate adenocarcinoma (NCT 02285101), as well as in a combinatorial setting with chemotherapeutics, i.e. oxaliplatin and capecitabine (NCT 02089633). Detailed information about therapeutic arginine-depleting enzymes is given in (Patil et al., 2016).

The development of resistance to ADT appears to be a serious problem and is suggested to mainly relate to an increase in ASS-1 expression upon treatment in initially ASS-1 deficient cancers. This is supported by systematic studies in ADT sensitive melanoma cells and their ADT-resistant counterparts developed via ADI exposure (ADI^r variants) (Long et al., 2013; Tsai et al., 2009). Here, the ADI^r variants showed enhanced ASS-1 expression which correlated with an increased c-myc transcription factor binding to the ASS-1 promotor. Resistance was also accompanied by an essential metabolic reprogramming manifested in enhanced AKT and reduced mTOR signaling. Also, alterations in glycolytic and glutaminolytic pathways (transporters and/or metabolic enzymes) were reported which more or less all seemed to be controlled via c-myc activity while HIF-1α was proposed as a negative regulator in this scenario (reviewed in Feun et al., 2015 and Kuo et al., 2010). Amongst others, metabolic reprogramming rendered the cells more susceptible to glutamine inhibitors, indicating that multi-metabolic combinatorial targeting might be a more efficient

innovative therapeutic strategy for these cancers. Such treatment options are of particular interest since the antitumor activity of arginine deprivation monotherapy in animal models and clinical trials seems lower as expected from *in vitro* data. This might be due to the putative support of ASS-deficient cancer cells by surrounding ASS-positive stromal cell compartments, but may also result from the lack of reflection of the *in vivo* situation in the majority of the *in vitro* assays applied earlier. Indeed, cancer cells in three-dimensional (3-D) spheroid culture were found to be less susceptible to single amino acid deprivation than the same cells grown in monolayer culture. Also, utilization of citrulline as arginine precursor in the 3-D assay seemed to better reflect the *in vivo* situation (Vynnytska-Myronovska et al., 2012 and 2013). By using such 3-D assays, we recently proposed the combination of ADT with the arginine analog canavanine plus irradiation as a new treatment strategy resulting in massive radiosensitization of cancer cells, apoptotic cell death and/or ER stress responses (Bobak et al., 2016; Vynnytska-Myronovska et al., 2012, 2013 and 2016; Kurlishchuk, 2016).

Successful and efficient depletion of arginine in the blood by the proposed enzymotherapeutical approach (ADI or arginase) is critical for treatment outcome. Monitoring of arginine levels in plasma samples of animals and patients has thus been an integral part of several *in vivo* studies (Ensor et al., 2002; Izzo et al., 2004; Kelly et al., 2012; Mauldin et al., 2012; Stone et al., 2012a) although no standardized routine protocol is proposed yet. The particular challenge as well as an easy handling procedure for identifying and quantifying arginine, its precursors and analogs in biological matrices is therefore highlighted in chapter 6.

2.4 Methionine uptake for cancer diagnosis and therapy

The enhanced dependency of cancer cells on exogenous methionine (Fig. 2) was initially described in 1959 for subcutaneously transplanted Walker carcinosarcoma xenografts in Sprague-Dawley rats fed over a period of 5 days with specific AA-supplemented or -deprived forage solutions (Sugimura et al., 1959). This was later confirmed by Halpern and colleagues in a first systematic *in vitro* study demonstrating impaired growth of human monocytic leukemia cells, rat carcinosarcoma cells and mouse lymphatic leukemia cells when medium was methionine-deprived but supplemented with the methionine precursor homocysteine (Halpern et al., 1974). A particularly high demand for methionine was in the following reported for cancer cells of different origins including human lung adenocarcinoma and acute lymphoblastic leukemia (Kreis and Goodenow, 1978), W-256 rat breast carcinoma cells (Hoffman et al., 1978), rat sarcoma and murine leukemia cells (Koziorowska et al., 1980) and others. Soon thereafter, it was discovered that not all human tumor cells depend on methionine supply with two studies in a panel of human tumors reporting relative methionine-independence for 12/23 and 17/17 cases, respectively (Judde and Frost, 1988; Mecham et al., 1983).

Despite extensive research, the underlying mechanism for both, loss of dependence and enhanced demand for methionine, are still in debate. The latter is to some extend caused by the high methionine uptake in actively proliferating cells. In addition, several metabolic deficiencies have been proposed. These include (i) defects or reduced activities of the enzyme MS/MTR (methionine synthase / 5-methyltetrahydrofolate-homocysteine methyltransferase) and its co-factor vitamin B12 which catalyze the synthesis from homocysteine to methionine and modulate the folate cycle, (ii) enhanced methionine transporter activity, and (iii) last but not least defects in the methionine salvage pathway - especially loss of MTAP (methylthioadenosine phosphorylase), (Fig. 3) (reviewed in (Cavuoto and Fenech, 2012; Cellarier et al., 2003)). The elevated uptake of methionine in tumors was used to develop [^{11}C]-methionine (MET) tracers for positron emission tomography (PET), namely [*S-methyl*-^{11}C]-methionine (Comar et al., 1976) and [1-^{11}C]-methionine (Bolster et al., 1986). These are now in clinical routine for diagnostics of brain cancers as a superior alternative to 2-deoxy-2-[^{18}F]-fluoro-D-glucose which has limited value in brain due to the high basic glycolytic flux in this organ resulting in an unfavorable normal tissue background (reviewed in (Glaudemans et al., 2013)). Several clinical studies are ongoing to also verify the proposed prognostic value of [^{11}C]-methionine-PET imaging for the prediction of progression-free survival in primary brain tumors (Yoo et al., 2015) and recurrent malignant gliomas (Jung et al., 2016) including our own clinical trial which is aimed at early detection of recurrence (NCT01873469).

Methionine-restricted diets were tested as therapeutic strategy in combination with chemotherapy in clinical phase I studies with metastatic cancer (Epner et al., 2002), metastatic melanoma and recurrent glioma (Durando et al., 2008; Thivat et al., 2009) and metastatic colorectal cancer (Durando et al., 2010). The interventions were generally well tolerated by the patients and could lead to partial response or stable disease but larger study cohorts are missing. It was shown that methionine deprivation leads to >90% depletion of SAM and MTA as well as a modest depletion of SAH (Tang et al., 2015). Also, epigenetic changes due to the missing main methyl-group donor under dietary conditions were identified and linked to methionine-specific gene expression (Tang et al., 2015). Dietary methionine withdrawal is also reinforced due to the finding that increased uptake of methionine in GBM was shown to alter the SAM/SAH ratio which in turn critically modified the DNA, RNA and protein methylation pattern towards a more aggressive epigenetic profile (Palanichamy et al., 2016).

A new therapeutic approach to reduce methionine represents methioninase which showed efficacy against various cancers in mouse models and was tested in macaque monkeys and in a pilot phase I trial presumably leading to best results in sequential combination therapy (reviewed in (Hoffman, 2015)). The original enzyme methionine-γ-lyase derived from

Pseudomonas putida degraded methionine but was as other bacterial methioninases instable in serum and highly immunogenic. Amongst others, a recombinant cystathionine-γ-lyase with specific methionine-degrading activity was engineered and PEGylated more recently which efficiently reduced serum methionine in mice from > 100 µM to < 5 µM and was much less immunogenic (Stone et al., 2012b). Monitoring plasma methionine in a time-dependent manner is critical not only to guarantee sufficient, therapeutically relevant reduction of physiological methionine during diet (Durando et al., 2010; Epner et al., 2002; Thivat et al., 2009) but also to ensure that this essential amino acid does not drop below a health-threatening threshold. The latter is particularly relevant when considering delicate enzymotherapeutic treatment approaches because methionine in contrast to asparagine and arginine is not sufficiently *de novo* synthesized in normal cells, i.e. even transiently undetectable or low methionine levels could lead to severe side effects (Durando et al., 2010; Thivat et al., 2009) (physiological AA concentrations see Fig. 2).

2.5. Clues from cancer metabolic profiling and conclusion

Metabolomics approaches were frequently applied to study the behavior of bacteria and yeast under nutrient-deficient conditions. However, similar investigations focusing on cancer versus normal (human) cells are still rare. Steady-state analysis of the tumor metabolome revealed a general higher need for amino acids in several entities (Hirayama et al., 2009; Kami et al., 2013). Proteinogenic AAs as well as some important metabolites were analyzed via liquid chromatography mass spectrometry (LC-MS) in an *in vitro* study of ovarian OVCAR-8 tumor cells depleted for asparagine by exposure to L-asparaginase. (Purwaha et al., 2014) (see also chapter 3). The authors revealed that asparagine was degraded by L-asparaginase extra- and intracellularly within seconds while the intracellular concentration of all other measured AA, increased immediately after the onset of treatment. Accordingly, the mechanism of these cancer cells to adapt to the asparagine-deprived environment seems complex, and interconnections between different metabolic pathways may thus affect treatment outcome (Figure 3). LC/MS-based metabolomics was also recently applied for comparative metabolic profiling in primary and established glioblastoma cells, glioblastoma tissues, and normal astrocytes (Palanichamy, 2016). Here, methionine, tryptophan, 5-methylthioadenosine (MTAP) and kynurenin turned out to be differentially regulated in glioblastoma cells as compared to normal human astrocytes. Methionine was found indispensible for tumor cell growth and survival. Furthermore, the increased methionine consumption altered the SAM:SAH (S-adenosyl-L-methionine:S-adenosyl-L-homocysteine) ratio which reflects the methylation potential of the cells as SAM donates methyl groups to DNA, RNA and proteins. In another comprehensive study, Tang and coworkers (2015) monitored changes in RNA expression of MCF-7 breast cancer and PC3 prostate cancer cells upon withdrawal of different, selected single amino acids. They could show that most

amino acids with the exception of glycine triggered a common transcriptional response that included specific ER stress response and cell cycle regulating genes. Beside heterogeneity, methionine deprivation resulted in particularly strong transcriptional effects which depended on the biosynthesis of creatine. The latter reduced SAM level under methionine-deprived conditions and lowered the histone methylations. This effect was abrogated upon simultaneous depletion of arginine or glycine (sources of creatine biosynthesis) indicating that the deprivation of multiple amino acids may not be superior to single amino acid withdrawal, and that combinatorial anti-cancer treatment strategies based on enzymotherapeutic and dietary amino-acid reduction have to be carefully validated in pre-clinical settings. Altogether, the authors describe a delicate crosstalk between methionine, arginine and glycine via the correspondent creatine biosynthetic pathway (Tang et al., 2015) (Figure 3).

In summary, different promising AA deprivation strategies are subject in preclinical and clinical cancer research and treatment. For clinical application of AA deprivation in patients, monitoring of plasma AA concentrations before and during dietary or enzymotherapeutic AA depletion should either be an inherent component for the evaluation of treatment success or alternative readouts relating to the level of AA deprivation have to be identified and validated. Towards this end, the method of choice should be a fast and reliable routine procedure with high sample throughput and sufficient sensitivity as well as specificity. It should be inexpensive and automatable with a simple sample preparation procedure. Choosing the appropriate technology for this purpose requires a close examination of the various classical and modern analytical tools for AA quantification on the market with their potential, advantages, problems and limitations as depicted in the following chapters.

3. A historical overview: Methodological pre-requisites in AA analytics

3.1 Basics: Electrophoresis versus chromatography

AA analysis is always performed in two steps – chromatographic or electrophoretic separation of the single AA from complex matrices such as biological fluids or protein hydrolysates followed by detection. For both processes, the most representative methods will be presented with a short historical overview.

Electrophoretic separation of differently charged AAs along an electric field is an integral method in molecular biology and shall thus be briefly outlined. The technologies' track record in AAD started in the late 1940s when the methodology initially developed by Arne Tiselius (1937) was successfully applied in preparative AA fractionation (Butler and Stephen, 1948). Classical electrophoresis using carrier materials such as paper or gel preparations, where the AAs are visualized via ninhydrin or other stainings, can be utilized but is less sensitive

than other AAD technologies and is in principle more common for the separation and semi-quantitation of proteins. Capillary electrophoresis (CE) became popular particularly after the pivotal work of Verheggen, Mikkers and Everaerts (1977) and was further developed for standardized AA and peptide analytics shortly after (Jorgenson and Lukacs, 1981). In CE, the derivatization process necessary for detection can run before, during or after separation with an in-capillary setting being preferential for handling (Tian et al., 2014). CE is a quite attractive tool for specific applications due to low costs, short separation times and easy sample management, and was therefore suggested as an alternative for the newborn screening (Jeong et al., 2013). These and other advances in CE for AAD as well as its coupling to MS have been highlighted in two recent reviews (Perez-Miguez et al., 2016; Poinsot et al., 2010). However, since CE has a relatively poor injection precision with limited repeatability, and artefacts due to frequent sample-matrix interactions, our essay will further focus on chromatographic methods for AA separation.

Chromatography as a laboratory method for AAD is irretrievably linked to the pioneering work of both the American chemist and geologist David T. Day and the Russian botanist Mikhail Tswett at the beginning of the last century. Day's (1859-1925) special interest as a geologist was the study and survey of oil shale deposits. He proposed that the varying composition of mineral oils from different deposits, while originating from a common source of organic material, depended on fractional filtration by surrounding minerals (Day, 1897). Arguing that the geological process could be reproduced in the laboratory, he tested this hypothesis by passing oil through fuller's earth (aluminum silicate), achieving separation into fractions of light petroleum, heavier oils, and petroleum jelly (Day, 1903). Beyond the industrial application of his discovery, he was also aware of its analytical power and stated that it would be possible "to characterize oil by the correct percentage of components hydrocarbons [...]." In contrast to Day, Tswett (1872-1919) was concerned with the analysis of biological samples, namely chloroplast pigments. In order to simplify pigment extraction procedures, he evaporated leaf extracts over paper strips by adding solvents and monitored the behavior of the adsorbed pigments (Tswett, 1906a). Discoloration was dependent on the solvent, with ether only solvating carotene while alcohols led to the total elimination of pigments from the paper. He later changed the absorption material from paper to inorganic compounds with a preference on calcium carbonate ($CaCO_3$). Thereby, he observed a certain adsorption sequence depending on the individual pattern of substance displacement. "When a petroleum ether solution is filtered through a column of adsorbent […], the pigments are resolved, according to the adsorption sequence, from top to bottom into various colored zones, since the more strongly adsorbed pigments displace the more weakly adsorbed ones […]." Tswett named the method *differential adsorption chromatography* (Tswett, 1906a) and described a laboratory process based on his discovery (Tswett, 1906a, b) which is still in use

in almost every chemical laboratory around the world. He continued to publish numerous papers in the field, most of them in Russian and thus inaccessible to a broader scientific community. This resulted in a 25-year latency period before the technology was internationally recognized.

The next notable progress in chromatography was documented in 1931 when Richard Kuhn, Edgar Lederer and Alfred Winterstein described the separation of isomeric compounds of carotene and xanthophylls (Kuhn and Lederer, 1931; Kuhn et al., 1931). Tswett's basic methodology was further improved by the development of both silica partition chromatography (Gordon et al., 1943; Martin and Synge, 1941) and paper partition chromatography (Consden et al., 1944), which combined a solid carrier with two liquid phases, i.e. water and an organic solvent. This facilitated the separation of structurally similar compounds, making complex biological samples amenable to analysis. Amongst others, Martin and Synge (1941) introduced the concept of theoretical plates, while Consden and coworkers defined the symbol R_f (retardation factor) to denominate the movement of an analyte relative to the liquid front (Consden et al., 1944). Based on these fundamentals, detection and separation techniques for increasingly complex matrices were developed; the most typical ones are delineated below.

A simple and cheap approach is thin layer chromatography (TLC) which was established for AAs in the 1950s (Mutschler and Rochelmeyer, 1959). Here, AAs are separated on a membrane consisting of resin material; the detection needs a chromophore or coupling to MS (Abu-Rabie and Spooner, 2009). The technique is easy in handling but suffers from quantification inaccuracies and is thus utilized only in qualitative or semi-quantitative analyses these days, in particular for enantioselectivity. An overview on recent applications as well as a comparison to HPLC and GC is given in (Dolowy and Pyka, 2014; Tanwar and Bhushan, 2015).

3.2 From liquid to gas chromatography

In liquid chromatography, three major separation techniques must be mentioned with respect to AAD: (i) ion chromatography (IC), (ii) reversed-phase (RP) and (iii) hydrophilic interaction chromatography (HILIC), usually performed as HPLC with a higher separation efficiency. IC is the traditional method in AA analysis based on the separation of polar analytes by their varying affinity to a stationary ion exchanger (Mansour et al., 2013; Williams, 1986). While ion exchange as phenomenon was already described in the mid-19th century (Way, 1850 and 1852), and synthesis of the first ion exchange resins was published in 1935 by Adams, it was not until the work by Small, Stevens and Baumann in 1975 that IC gained real foothold in analytical chemistry. The authors described for the first time the use of sub-micrometer particles in IC and directly coupled the separation to a conductivity cell as detector, thereby

reducing column size and analysis time. Another important step in the analysis of organic compounds by IC was the development of a pulsed amperometric detector in 1983, which finally allowed the detection of AAs and carbohydrates (Rocklin and Pohl, 1983). The basics of contemporary RP chromatography were described in 1950 by Howard and Martin as a development of partition chromatography (Howard and Martin, 1950). Here, silica gel was modified by dichlorodimethylsilane to enhance the interaction of hydrophobic compounds with the solid phase. The combination with a non-polar mobile phase of acetone-paraffin allowed the first separation of long-chain fatty acids. The esterification of silica with various hydrophobic groups was patented shortly thereafter (Iler, 1953), enabling the synthesis of column material with highly selected separation properties. The term hydrophilic interaction liquid chromatography was first used in a review by Alpert in 1990 to describe the combination of ion exchange resins and RP solvents (Alpert, 1990). In contrast to RP, the solid phase is modified with polar groups like amino, amide, cyano or hydroxyl functionalities, and the gradients start with a high percentage of organic modifier (Guo, 2015; Tang et al., 2014). Elution is achieved gradually by increasing the water content of the solvent. Therefore, HILIC is orthogonal to RP-LC showing higher separation efficiency for polar analytes. Since HILIC and RP can use the same solvent system, the deployment of both systems in a two-dimensional (2-D) method is feasible (Stoll et al., 2008). The term Amino Acid Analysis (AAA) has been assigned to an AAD process based on an automated cation-exchange chromatography (specific HPLC) with ninhydrin postcolumn derivatization which is used since the 1960s and comprehensively described in (Inglis, 1964; Kaspar et al., 2009a; Kellner et al., 1994; Starbuck and Busch, 1962).

As an alternative, analysis of AAs can be performed by high resolution capillary GC (*syn.* gas-liquid chromatography (GLC)). GC/GLC per se is highly efficient, has good sensitivity and is characterized by particularly high speed and flexibility with respect to the deployed capillary. Furthermore, the instrumentation costs are moderate. The main limitation of the approach is the need for derivatization which usually involves laborious and time-consuming procedures resulting in a loss of the GC advantage for high speed. GC was initially applied to the analysis of gases and vapors. The work of Martin & Synge (1941) and James & Martin (1952) then launched GC as a highly versatile technology applicable to a plethora of gaseous samples, liquid solutions and volatile solids; for non-volatile samples of interest, techniques such as pyrolysis GC or derivatization GC were established. Pyrolysis GC is an approach based on the thermo-chemical cleavage of non-volatile probes prior to their entry into the column/ capillary where the smaller, more volatile decomposition products are separated (Irwin and Slack, 1978). Ulehla was the first to study the pyrolysis of potassium salts of 19 AAs with 13 of them giving appropriate pyrograms for analysis (Ulehla, 1960). Further attempts at identifying the pyrolysis fragments from AA monomers were made by Kanomata

& Mashiko (1966) and by Winter & Albro (1964). A logical extension of this technique was the early interfacing of pyrolysis GC with mass spectrometry (MS) for better identification of the decomposition products (Voellmin et al., 1966). Based on the pioneering work of Golay (1957) and Holmes & Morell (1957), GC-MS finally proved to be one of the most sophisticated and powerful analytical tools also for AAD. However, derivatization GC became more important for GC-MS as sensitivity, specificity and efficiency of AA analysis could be tremendously improved.

In order to make AAs amenable to GC separation, they have to be quantitatively converted into less polar and, essentially, volatile derivatives. The most common derivatization protocols comprise the formation of perfluoroacyl alkyl esters (Gehrke et al., 1968; MacKenzie and Tenaschu, 1974) or silylation reactions replacing active hydrogens by alkyl silyl groups (Chaves Das Neves, 1982; Gehrke et al., 1969). However, their methodological limitations include high reagent costs, laborious pre-analytical and synthetic processes requiring reagent removal and solvent exchange as well as the sensitivity of reagents and derivatives to moisture. Indirect alkylation of AAs in aqueous solutions via alkyl chloroformates in the presence of an alcohol and pyridine or 3-picoline as catalyst was introduced by Hušek and co-workers in the 1990s (Huang et al., 1993; Hušek, 1991) and turned out to be a very powerful approach. Here, the AA react in an incredibly rapid single-step procedure allowing for simultaneous acylation of the amino group and esterification at the carboxyl function at room temperature (Hušek, 1998). The derivatives can be easily extracted with an organic solvent, and an aliquot is then directly injected into the GC-MS system. The approach can be individually tailored to specific analytical aims, e.g. the analysis of specific biological/ physiological specimen (Kaspar et al., 2008; Zahradnickova et al., 2007), analysis of sulphur-containing AAs (Pietzsch et al., 1997a), analysis of non-protein/modified AAs (Hušek et al., 2008; Pietzsch et al., 2004), stable isotope ratio analysis of AAs (Pietzsch et al., 1997b) or chiral speciation of AAs (Simek et al., 2012). This is achieved by using different alkyl or fluoroalkyl chloroformates, e.g. methyl, ethyl, propyl, or pentafluoropropyl chloroformates, and various alcohols such as ethanol, propanol or trifluoroethanol which determine the formed esters. For MS, standard electron impact ionization (EI) can be used which is easier to maintain than chemical ionization (Cao and Moini, 1995; Pietzsch et al., 1995); ionization strategies will be further discussed in Chapter 4. Moreover, alkyl/ fluoroalkyl chloroformate derivatization of AAs can be adopted for liquid chromatography (LC) (Hušek et al., 2016). On the other hand, this approach with its variations related to the employed chloroformate and alcohol is characterized by an excellent sensitivity with a detection and quantification threshold in the low micromolar range as well as broad analytical/ calibration ranges (Kaspar et al., 2009a; Pietzsch and Pixa, 1998).

One of the limitations of this analytical technique is the challenge to analyze arginine as the guanidine moiety is unstable. The analysis of arginine and chemically related molecules by GC-MS constitutes a more general problem as reviewed recently in a highly informative article by Martens-Lobenhoffer & Bode-Böger (2014). Indeed, two major prerequisites, vaporability and thermal stability of the derivatives, are often not given or achieved for all analytes of interest. Preparative solutions were described only for selected molecules or matrices, e.g., for dimethylarginines (Tsikas et al., 2003 and 2011) or for arginine plus ornithine (Yoon, 2013), but Martens-Lobenhoffer and Bode-Böger clearly illustrate that LC-MS-based analysis of arginine and its related substances still remains the 'gold standard' (see Chapter 6).

4. Competing or complementary: Classical and modern AAD at a Glance

4.1 AAD technologies - an overview

HPLC is probably still the most widely used analytical method for AAD. Here, the detection of separated AAs based on ultraviolet (UV) or fluorescence (FL) light excitation has been standard for many years. Notably, peptides and proteins exhibit strong UV absorption in the range of 190-230 nm due to their numerous amide bonds (Goldfarb et al., 1951), but absorbance of single AAs is limited to the aromatic AAs phenylalanine, tryptophan and tyrosine which are typically detected at 280 nm. The only AA showing real strong intrinsic fluorescence upon excitation is tryptophan which emits in the range of 335-380 nm with high intensity (Eaton, 1988). UV/FL-based identification of the other AAs requires the introduction of a chromophore into the AA molecules. Historically, ninhydrin and phenyl isothiocyanate (PITC) have been among the most famous AA-derivatization reagents (Rutherfurd et al., 2009).

First described by Ruhemann in 1910, ninhydrin as AA-reactive compound is forming a purple color (Ruhemann's purple) after reaction with primary amines or a yellow complex when reacting with secondary amines (Ruhemann, 1910a, b). Amongst others, the ninhydrin approach was used in the fundamental study by Consden, Gordon & Martin (1944) referenced earlier in this review as well as in the first AA analyzer described by Moore & Stein (Moore and Stein, 1948; Stein and Moore, 1948). After derivatization, AAs with primary amine reactions are visualized at 570 nm, while those with secondary amines like proline and hydroxyproline can be detected at 440 nm (Friedman, 1974; Troll and Cannan, 1953; Yemm and Cocking, 1955). Besides its use as post-column derivatization agent in chromatographic AA analysis, ninhydrin is employed as fingerprint marker in forensic science (Odn and von Hofsten, 1954) and as an important control reagent in solid phase peptide synthesis (Kaiser et al., 1970). Another option of AA derivatization is the use of isothiocyanates (Santa, 2010).

Best known is perhaps PITC - also called Edman's reagent because it is most commonly associated with the hydrolytic sequencing of peptides by Edman degradation (Edman, 1950). While yielding chemically very stable derivatives, the main drawback of PITC is its lack of fluorescence emission, leading to relatively high detection limits. For a detailed discussion on PITC-derivatization of AAs see (Sherwood et al., 1990). Today, the most popular compound for FL-detection of AAs is *o*-phthalaldehyde (OPA) (Klein and Linser, 1932; Zimmermann, 1930; Zuman, 2004). Derivatization is performed at basic pH (8-11) with organosulfur compounds like ethanedithiol (Carducci et al., 1999), 2-mercaptoethanol (Chen et al., 1997) or 3-mercaptopropionic acid (Terrlink et al., 1994). While achieving excellent sensitivity with picomolar Limit(s) of Detection (LOD), derivatization with OPA yields instable products which degrade into non-fluorescent compounds, making the analysis of complex biological matrices difficult (Garcia Alvarez-Coque et al., 1989). The repertoire of AA derivatization reagents has been broadened over the years as exemplified in Tab. 1; all of these compounds are able to form AA derivatives that allow detection in the picomolar range.

The most exciting technological progress of the past decades in AAD is clearly associated with mass spectrometry (MS). During MS analysis, ions are formed at the MS interface and transferred into a gas phase. For the determination of target molecules, ions become accelerated in an electric field, are transmitted to the analyzer and finally sorted by their *m/z* (mass-to-charge) ratio. Analytes can deliberately be fragmented during this procedure depending on the type of MS. Historically, the development of GC for separation and detection by MS went hand-in-hand (see Chapter 3) (Biemann and Vetter, 1960). After the initial publications on GC in 1952 (James and Martin, 1952; James et al., 1952), the first successful application of analytical MS was already documented in 1956 by Drew who described the separation and subsequent analysis of hexane, propyne (methylacetylene), propylene and allene isomers (Drew et al., 1956). State-of-the-art GC-MS is mainly based on either electron ionization (EI) or chemical ionization (CI). Ionization by electron bombardment was first described by Dempster almost a century ago for the analysis of cathode rays and determination of isotopes (Dempster, 1918; Dempster 1921). The same fundamental technique is still applied in GC-MS, with a hot filament serving as electron source and allowing the coupling of GC and EI with time of flight measurements (Gohlke, 1959). The direct ionization by electron bombardment in EI constitutes a 'hard' ionization method leading to strong fragmentation of the analyte. Hence, 'softer' methods that keep the analyte molecule intact have been of great interest for the analysis of biological samples. In 1966, Munson & Field described the use of methane as a reaction gas in the ionization process and defined the process as chemical ionization (Munson and Field, 1966). Operationally, the reaction gas is ionized first and the secondary species of ionized gas then leads to the ionization of analyte by proton or hydride transfer. In this case, electron impact on the analyte

molecules is negligible due to the high excess of reaction gas, and fragmentation patterns are mostly limited to reactive centers (Arsenault et al., 1970).

4.2 State-of-the-art: GC-MS and LC-MS

The developmental progress towards widely applicable LC-MS has clearly been more complex. The shift in paradigms initiated by the increasing availability of commercial LC-MS systems shall be illustrated by two quotes from publications in *Electrophoresis*. In the ongoing series on capillary electrophoresis: Poinsot claimed in 2003 *"In fact it [MS] is not often used because MS is expensive, and, because of the low-molecular-mass of AAs, the detection is not sensitive."* (Poinsot et al., 2003); thirteen years later, it was stated in the same series, *"Thanks to its relevant advantages, providing both universality and selectivity, high sensitivity, and rich information content, MS is the most powerful detection system for CE, CEC and other separation techniques."* (Kašička, 2016). Initial problems with the coupling of LC procedures with MS primarily occurred at the interface of the two systems where the continuous liquid flow from the LC had to be linked to the MS gas-based detection technique in vacuum. This dilemma was solved by the development of atmospheric pressure ionization (API) (Carroll et al., 1974; Horning et al., 1973) and electrospray ionization (ESI) (Yamashita and Fenn, 1984), both allowing mass spectrometric real-time analysis of the chromatograms.

One development in API is atmospheric pressure chemical ionization (APCI). Here, the LC flow is directly injected into a vaporization tube, where it hits a high-pressure stream of nitrogen leading to nebulization. The solvent is then evaporated at temperatures of 400 °C and directed towards a high voltage corona needle were discharge and ionization processes take place. This allows two-step ionization similar to CI, i.e. high voltage induces first ionizing solvent molecules in the gas which then ionize the analytes (Bruins, 1991). The latest API methodology is atmospheric pressure photo ionization (APPI) (Robb et al., 2000). The experimental strategy is similar to APCI but includes a gas-phase dopant like toluene, acetone, anisole or chlorobenzene which enhances the photoionization when added to the drying gas during evaporation. The evaporated LC flow then passes a discharge lamp emitting 10 eV photons which ionize the dopant molecules forming a large number of free radicals and molecular ions. Subsequently, analyte molecules are ionized by the dopants through electron or proton transfer (Raffaelli and Saba, 2003). In contrast to these and other API techniques, ESI is based on the direct ionization of analytes from the solvent stream. The solvent stream exiting a capillary forms a fine aerosol when energized by high voltage. As the solvent evaporates from the droplets, electrostatic repulsion overcomes the surface tension leading to Coulomb fissions until all solvent is evaporated. At this point, charge carriers from the solvent like protons or metal cations are transferred, and only the charged analyte molecules remain (Iribarne, 1976). When using ESI, special attention must be drawn

on the solvent composition because ion pairing and derivatization agents are critical for ionization performance (Garcia, 2005; Krusemark et al., 2009). Beyond AAD, ESI has been successfully applied to softly ionize large biomolecules (Fenn et al., 1989). In any case, the choice of ionization technique mainly depends on the polarity of the analyte, i.e. ESI is well suitable for polar molecules but less effective for the analysis of non-polar compounds where APCI and APPI might be advantageous (Niessen et al., 2006).

Independent of the ionization method of choice, MS has become the predominant mode of detection in metabolomics and for AAD in particular. With GC-MS and LC-MS, two separate techniques are widely used in the field. However, it is still a matter of debate which of these techniques is superior and can be considered as state-of-the-art "gold standard". The number of actual comparative studies for both analytics platforms is surprisingly limited given the rapidly expanding field of metabolomics.

A first conclusive study on this issue was published by Kaspar et al. (2009a). Here, AAD in two batches of urinary samples via a commercial AA analyzer was compared to (i) a GC-MS method using propyl chloroformate derivatization combined with isotope-labeled internal standards (see also Chapter 5), and (ii) an interesting LC-MS/MS method employing for the first time isobaric tagging by iTRAQ® in the analysis of physiological AAs. Separation and analyses were performed on commercially available systems (*AA analyzer*: Biochrom 30; *GC-MS*: Agilent 6890/5975 with a ZB-AAA column; *RPLC-MS/MS*: Agilent 1100 Series LC system with an Applied Biosystems C_{18} column coupled to an Applied Biosystems API 3200 MS with one-transition multiple reaction monitoring (MRM)). Total run times varied between 130 min for the AA analyzer and 20 and 25 min for GC-MS and iTRAQ®-LC-MS/MS, respectively. The AA analyzer also required higher concentrations as Lower Limit of Quantification (LLOQ; 2-3 µmol/L) compared to GC-MS (0.3-30 µmol/L) and iTRAQ®-LC-MS/MS (0.5-50 µmol/L). LC-MS/MS was superior in overall amenability of AAs covering 42 of 45 analytes, closely followed by the AA analyzer with 40. GC-MS only discriminated 26 of the analytes, i.e. detection was strongly limited by either thermic instability (Arg) or low vapor pressure (e.g. phosphoserine). AA analyzer and GC-MS were comparable in terms of reproducibility, with an average technical error (% TE) of 7.59 ± 4.96 % and 8.28 ± 6.64 %, respectively, for the second batch of samples. In the case of GC-MS, reproducibility was higher only if isotope-labeled internal standards were added (5.87 ± 3.59 % vs. 13.03 ± 8.31 % TE for AAs without standard, for which the nearest eluting IS was used as references). Despite intrinsically available isotope-labeled internal standards, iTRAQ®-LC-MS/MS initially performed worst with a TE of 30.38 ± 19.16 %. This could predominantly be attributed to an excess of MRM transitions acquired within one of four predefined time windows. The authors argued that this shortcoming could be alleviated by implementation of a new MRM schedule which exclusively monitors each AA within the time of its expected

elution. Both GC-MS and LC-MS/MS were thus considered as excellent alternatives to the AA analyzer, with GC-MS having the advantage of full automation, short run time and high precision, while iTRAQ®-LC-MS/MS made more AAs amenable to analysis. The methodology described in this study was later adopted in *AA Analysis: Methods and Protocols* (Dettmer et al., 2012).

An independent, more recent study on GC-MS and LC-MS performance in AAD was undertaken by Krumpochova and co-workers, who presented a detailed inter platform comparison of GC-MS, HPLC-MS, and HILIC-MS (Krumpochova et al., 2015). Separation and analyses were again based on the use of commercially available systems (*GC-MS*: Shimadzu GC-2010 gas chromatograph with a ZB-AAA column coupled to a GCMS-QP2010 Plus EI-MS; *RPLC-MS*: Agilent 1100 Series LC system with a Zorbax Eclipse XCB-C_{18} column coupled to an AC-Sciex API 3000; *HILIC-MS*: Shimadzu Nexera LC30 AD LC system with a Phenomenex Luna-NH_2 column coupled to a Shimadzu LCMS-8030 ESI-MS). Samples for GC-MS and RPLC-MS were subjugated to derivatization with propyl chloroformate using the EZ:faast™ amino acid analysis kit (Badawy, 2012). A method-specific optimization and determination of LODs, LLOQs, repeatability and reproducibility was first performed on a ^{13}C,^{15}N-labeled mixture of all 20 canonical AAs as quality control (QC) to facilitate inter platform comparison. Subsequently, run times (including column equilibration) of 7 min for GC-MS, 20 min for RPLC-MS and 45 min for HILIC-MS were accomplished.

None of the methods utilized in (Krumpochova et al., 2015) were able to achieve total baseline separation for all AAs of interest; GC-MS was unable to separate isoleucine/leucine and asparagine/methionine. An additional constriction in GC-MS was the invisibility of arginine due to thermal instability of the derivative. Separation of isobaric AAs was feasible via LC-MS, and further identification of non-baseline separated AAs was performed by SRMs in MS/MS. Comparable LODs were achieved with lower limits of 50 nM in GC-MS and ≤ 100 nM in RPLC-MS for all analytes. LODs in HILIC-MS differed for particular AAs and were approximately100 nM for cystine, glycine and threonine and in the range of 1 - 30 nM for all other AAs. In terms of accuracy, both LC-MS methods performed comparably well with intra-/inter-day accuracies of 90 - 120 % (RP) and 80 - 120 % (HILIC) using ^{13}C,^{15}N-labeled standards. GC-MS was more accurate with 99 - 105 % / 83 - 115 % intra-/inter-day accuracy. Linearity varied between 0.05 - 200 µM (GC-MS), 0.1 - 100 µM (RPLC-MS) and 0.03 - 100 µM (HILIC-MS). The use of ^{13}C,^{15}N-labeled internal standards allowed to expand the LLOQ six-fold for GC-MS, ten-fold for RPLC-MS and three-fold for HILIC-MS. After analysis of the QC sample, an animal tissue hydrolysate (Primatone®, Sigma-Aldrich) - containing all proteinogenic AAs except arginine, glutamine and cysteine - was used as test sample. All three platforms showed similar standard deviations; however, a general

underestimation of yield delivered by HILIC-MS relative to GC-/RPLC-MS was noted. In addition, RPLC-MS failed to fully separate valine and norleucine with the internal standards of the EZ:Faast kit. Analysis of valine was possible though, when using ^{13}C,^{15}N-labeled standards. Due to the similar performance of all three platforms, intrinsic matrix effects could either be neglected or were directly corrected by using ^{13}C,^{15}N-labeled standards. Hence, the authors concluded that all three methodologies are suited for the reproducible analysis of AAs, with the GC-MS setup being most precise and considered best for a targeted approach of selected AAs. HILIC-MS as the most versatile technique can separate a larger pool of hydrophilic compounds and might thus be superior for broader metabolomics studies beyond AAs. RPLC-MS, while not particularly outstanding in any category, was nevertheless the only method capable of quantifying all AAs in both the QC sample and the animal tissue hydrolysate.

In conclusion, both GC-MS and LC-MS appear to perform quite well in AAD. GC-MS generally achieves excellent accuracies but derivatization of AAs is essential. Combined with the high temperatures required for analysis, this reduces the number of AAs amenable to analysis. In contrast, the full spectrum of AAs is accessible by LC-MS but at the cost of higher analysis time and often only by employing derivatized AAs to enhance separation efficacy. Several newer studies have succeeded in reducing analysis time to 12 - 15 min for LC-MS of underivatized AAs by employing 2-D LC and MRM methods (Le et al., 2014; Zhou et al., 2013 and 2015). With a three minute (3AA) separation procedure, Nemkov et al. (2015) recently published the shortest protocol for highly sensitive and reproducible AAD and quantification from biological samples via LC-MS using an ultra-high performance liquid chromatography (UHPLC) (Ultimate 3000, Thermo); here, separation was performed on an XB-C18 column with 1.7 µm particles by isocratic elution with AcN/H_2O (5:95, 0.1 % CH_3COOH). Coupled to a QExactive MS (Thermo, San Jose, CA, USA), this setup allowed the detection and quantification of 35 AAs from three different AA standards (SD1-3, Phenomenex, Torrance, CA, USA) as well as the analysis of AAs from rat tissue samples. These results were consisted with parallel, gradient-based measurements on standard HILIC-MS (15 min analysis time) and an amide HILIC-MS (23 min analysis time) system, surpassing these standard systems in terms of signal to baseline ratios, LOD and LLOQ for the standards and delivering comparable results for relative quantitation of pancreatic cancer cell extract samples. Limitation to the 3AA method was the relatively high influence of matrix effects on linearity requiring the determination of matrix-dependent linear ranges for quantification in future applications. Nonetheless, this derivatization-free method might indeed be useful for high-throughput AAD as stated by the authors, especially for routine samples in biomedical research.

5. In focus: Potential and challenges of LC-MS based AAD

5.1 Sample preparation and handling

Despite high acquisition and maintenance costs, the combination of LC with (tandem) MS (LC-MS) has increasingly been utilized for AAD in recent years due to its high sensitivity and accuracy. LC-MS has become the gold standard for pesticide and drug residue analysis in environmental, food and clinical chemistry (Fang et al., 2015; Shushan, 2010; Thornalley and Rabbani, 2014; Van Eeckhaut et al., 2009). While the discrimination of analytes in UV/FL-HPLC is exclusively based on the retention time, MS can differentiate even overlapping peaks by the unique fingerprint of fragmentation pattern of each molecule as detailed in chapter 3. LC-MS can be combined with optical detectors for internal cross-check (Armstrong et al., 2007; Kaspar et al., 2009a). As technical overview, we recommend reading of the excellent 2009 article by James Pitt, an expert in LC-MS analytics and newborn screening (Pitt, 2009). We will in the following paragraphs describe the process from sampling to AA quantification via LC-MS as drafted in Fig. 5 and address major pitfalls, some of which have briefly been mentioned in the previous chapters.

Spatiotemporal representative sampling is crucial for high quality AAD. AA quantification in human plasma, for instance, requires sampling in the morning because AA concentrations in the blood follow a circadian rhythm being lowest in the morning in the absence of overnight ingestion and highest in the afternoon (Feigin et al., 1967). The sample preparation protocol for LC-MS has to be adapted for the molecules of interest, the expected concentration range and the sample matrix composition. Biological matrices such as plasma, serum, whole blood, dried blood spots, urine, liquor or tissues usually contain phospholipids, fat and/or proteins and can be delicate in handling, in particular for low concentrated analytes. These matrix constituents impair AA separation, can increase the instrument pressure and block the column. Deproteinization of the samples is essential but not trivial. The application of sulfosalicylic acid is quite common for protein precipitation, even if this technique shows a poor AA recovery and overlapping peaks due to the rather high sulfosalicylic acid signal (Aristoy and Toldra, 1991; Liu et al., 2001). Alternatives to sulfosalicylic acid are various organic solvents such as acetonitrile and/or methanol which shall best be combined with ultrafiltration. Nonetheless, non-precipitable compounds such as fatty acids or phospholipids remain in the sample (Ghassabian et al., 2014; Joyce et al., 2016; Nemkov et al., 2015; Van Eeckhaut et al., 2009). Defatting of samples with low lipid content can be achieved if the deproteinization is performed at 4°C; for high lipid contents it is necessary to use unpolar solvent such as hexane or dioxane. Phospholipids can then be removed using commercially available solvent kits (Carmical and Brown, 2016). Sample preparation should further include an additional step to meet with putative matrix effects. Suitable methods are solid phase

extraction (SPE), the implementation of internal, stable isotope standards, and additional derivatization and/or filtration steps.

As detailed in chapter 4, many derivatization procedures are approved for AAD via HPLC. Is derivatization therefore mandatory? Indeed, the derivatization step which is required in HPLC is not obligate for LC-MS but can of course enhance the sensitivity of AAD (Santa, 2011). Major drawback and limitation for large-scale studies is the time frame required for the derivatization reaction with risk of error (Salazar et al., 2012). AA derivatization is carried out to convert hydrophilic AAs into more hydrophobic molecules for reversed phase chromatography and reduction of matrix effects (Santa, 2011). In LC-MS, it further aims at the introduction of ionizable groups improving the sensitivity, selectivity and accuracy of the measurement; the higher molecular weight of the derivate AA leads to an enhanced signal to noise ratio (Kaspar et al., 2009b; Rydberg et al., 2009; Salazar et al., 2012). Reagents exclusively developed for rapid derivatization of small samples via LC-MS are listed in Tab. 1. Further details and alternative reagents are described elsewhere (Kaspar et al., 2008; Santa, 2011).

5.2 Prospects and limitations of LC-MS without derivatization

For many AAs, LC-MS without derivatization is high-speed and convenient (Armstrong et al., 2007; Joyce et al., 2016). Chaimbault and colleagues were the first to describe the parallel analysis of 20 underivatized AAs with LC-MS in the late 1990ies (Chaimbault et al., 1999). Today, the addition of ion-pairing agents to the mobile phase is a common procedure to then also apply reversed phase columns for non-derivatized AA separation; Chaimbault used trifluoroacetic acid for this purpose (Chaimbault et al., 1999). In 2007, Armstrong et al. could in principle detect 25 AAs without derivatization since all of them are usually well ionized. ESI (see chapter 4) as method of choice for gentle biomolecule ionization and fragmentation was applied (Kulyk et al., 2015). For improving the specificity of the measurement, a MRM protocol was engaged that allows identification of specific precursor-to-product ion transitions (Armstrong et al., 2007). Nonetheless, a concentration step might be advised in case of low-concentrated analytes. Recently, a protocol for the sensitive detection of 18 proteinogenic AAs in natural water samples with reduced matrix effects was presented using SPE (How et al., 2014). Of note, homocysteine (HCy) which is relevant in methionine metabolism and associated with various diseases as highlighted in this review, was not included in previous AA panels – not because it has to be derivatized but due to other analytical challenges, e.g. the formation of dimers (homocystine) via disulfide bridges. The newborn screening highlighted in chapter 1, for example, contains homocysteine in some countries but not others, although adapted analytical protocols for easy sample handling and fast detection have recently been published (Chambers et al., 2014; Ghassabian et al., 2014).

Despite the technical progress, LC-MS measurements are still susceptible to interference requiring adequate troubleshooting strategies. Although run times are becoming shorter, long equilibration periods for the columns to remove the accumulated ion-pairing reagent limit sample high-throughput (Chaimbault et al., 2000). The general assumption that LC-MS allows to run 30 samples (or more) per hour has been questioned by unreliable, non-reproducible results (Matuszewski et al., 2003). Indeed, elimination or shortening of the intermediate equilibration steps may cause problems during analysis reflected by shift of the peaks, double or split peaks and alterations in peak shapes. Besides, matrix effects - first described by Kebarle & Tang (1993) - can manifest in similar signal falsification. The state of knowledge in matrix effects has been outlined earlier (Antignac et al., 2005; Matuszewski et al., 2003; Van Eeckhaut et al., 2009) but the underlying interrelations are still not completely understood (Fang et al., 2015). In brief, two substantial negative effects may be seen in LC-MS analysis of AAs in biological samples, namely ion suppression and ion enhancement. Both adversely affect the quality of readout (Cappiello et al., 2008). In this context, undesired interferences between co-eluting matrix and analyte at the ion source affecting analyte ionization as mentioned earlier and co-eluting matrix at the interface altering the pH are under discussion (Matuszewski et al., 2003; Van Eeckhaut et al., 2009). Here, the term matrix comprises either endogenous compounds from the sample matrix or exogenous compounds trespassing with sample preparation (Antignac et al., 2005; How et al., 2014; Van Eeckhaut et al., 2009). Samples undergoing ESI seem to be more frequently affected (Cappiello et al., 2008; Trufelli et al., 2011; Van Eeckhaut et al., 2009). Various strategies proposed to tackle these problems are summarized in Tab. 2.

6. LC-MS for monitoring specific therapy-related AAs: An example

The power of LC-MS for plasma AAs monitoring is increasingly appreciated in cancer research and therapy. Dietary or enzymotherapeutic depletion of selected (single) AAs are promising systemic treatment options for various malignant diseases as emphasized in chapter 2. We recently found arginine withdrawal - also termed arginine-deprivation therapy (ADT) - to enhance radiosensitivity in cancer cells with high arginine demand even if they were *per se* non-auxotrophic for this AA; co-exposure to low doses of the arginine analogue canavanine demonstrated tumor-selective efficacy in colorectal cancer models *in vitro* and supported or augmented the radiosensitizing effect (Vynnytska-Myronovska et al., 2012; Kurlishchuk et al., 2016). Current GC-MS methodology fails to reliably quantify arginine levels (Kaspar et al., 2009a; Krumpochova et al., 2015), and is thus not adequate for treatment monitoring neither in the experimental *in vitro* and *in vivo* settings nor in the clinical situation. LC-MS was considered as method of choice due to its high specificity and low

detection limits. For our own studies, we developed a 20-minute LC-MS/MS protocol for the simultaneous detection of the structurally related AA arginine, citrulline, ornithine and canavanine on the one hand (Fig. 6), and for methionine and homocysteine on the other hand, which is applicable to a variety of matrices such as culture medium, plasma and tissue (Kurlishchuk et al., 2016 and unpublished data; see Fig. 3 for information on AA pathways).

Proteinogenic arginine is an alkaline AA with a guanidine group; citrulline its non-proteinogenic precursor in the urea cycle carries a ureido instead of the guanidine group, and canavanine is a non-proteinogenic guanadinooxy structural analogue of arginine (Morris, 2002, 2006 and 2009) (Fig. 6). The chemical analogies of these AAs as well as the complexity of matrices of interest are challenging. Our protocol thus includes the addition of stable isotopes as internal standard for each analyte. Deproteinization is performed using an acetonitrile/methanole mix followed by an ultrafiltration prior to sample transfer into the MS vial. The filtration step is cost-intense but should not be omitted for the benefit of an informative chromatogram. Directly after running the optimized sample preparation procedure (protein precipitation, centrifugation, filtration), a classical ion pair reagent (trifluoroacetic acid, TFA) is applied as mobile phase. The chromatographical separation is adjusted to define the retention and elution times for each analyte. A Hypercarb column (Thermo Fisher) consisting of a porous graphite packing material as recommended for highly selective AAD with LC-MS (Chaimbault et al., 2000) is utilized due to its ability to efficiently separate polar compounds. A prerequisite for successful MS measurements is the solid and sensitive detection of analytes. Here, a triple quadrupole mass spectrometer (Agilent 6410) is used with optimized MS settings like fragmentor and collision voltage for all molecules of interest. Optimal values for adjustment were defined beforehand for each individual isotope-labeled standard by monitoring the signal along an incrementally changing MS voltage to set up the final MRM method. Calibration curves were prepared by recording isotope standards with different concentrations and plotting the concentration as function of the peak ratio. Based on that, analyte concentrations in unknown samples can now be determined from each individual peak area relative to the respective isotope standard as exemplified in Fig. 6. In our setting, the lower limit of detection is in the range of 50-100 nmol/l with an injection volume of 1-2 µl. The same limits apply to the detection of methionine whereas homocysteine quantification turned out to be more complex and less sensitive. By introducing an additional reduction step using dithiothreitol to efficiently convert homocystine back into its monomers, homocysteine can be analyzed with a detection limit of 0.5-2 µmol/l which is in the range published earlier (Hellmuth et al., 2011; Hempen et al., 2008).

Pitfalls in the development of the protocol included peak fronting by column overload and ion suppression problems. This was solved by decreasing the amount of accumulating nonvolatile TFA in the ionizing interface and reducing the injection volume to a maximum of

1-2 µl per injection. As expected, aqueous solutions were well suited as matrix but plasma caused problems reflected by shifted rkruseetention times and slightly split peaks for arginine due to increased matrix effects and tailing, eventually caused by secondary retention. Inline filters for column protection and internal isotope standards unmasked and minimized matrix effects. A word of caution though - peak broadening and splitting can also result from a damaged frit or a void in the column. Storage of the internal standard solutions can also be critical. A decrease in sensitivity can to some extent be compensated by adjustment of the MS setting (EMV) but the occurrence of non-isotope amino acid peaks in chromatograms of the presumably stable isotope standards is a clear *no-go*. Quality control is thus obligate.

In summary, the proposed LC-MS/MS protocol turned out to be a fast, reliable and suitable approach for the quantification of related AAs such as arginine, its precursors and putative analogs in biological samples and may thus be useful not only in basic and preclinical research but also in clinical routine.

7. Summary and Conclusions

Amino acid (AA) metabolism is important in health and disease. Thus, AAD is an integral part of basic and applied life sciences and associated disciplines including biotechnology, pharmacy and food chemistry. Standardized, easy handling tools for AA profiling are available or under development for various applications and are also in great demand in the field of biomedicine for the diagnosis and treatment of metabolic disorders. Amongst others, cancer should be considered as metabolic disease since carcinogenesis is accompanied by genetically and epigenetically driven alterations in central and intermediate metabolic pathways which open a window for novel theranostic concepts. Malignant cells can develop auxotrophies for non-essential and semi-essential amino acids and are often more susceptible to single AA withdrawal due to their high proliferative activity and metabolic enzyme deficiencies. The potential of AA metabolic targeting via systemic dietary and enzymotherapeutic strategies in cancer research and treatment can convincingly be demonstrated with three archetypes of proteinogenic AAs: asparaginine (non-essential), arginine (semi-essential) and methionine (essential).

The need but also challenges and limitations for analyzing the respective AA in biological fluids and solid materials such as plasma or tissue from experimental targeting studies and, in particular, in clinical routine for treatment monitoring are recognized. In this context, classical and highly sophisticated, complementary technologies are presented and compared including different GC-MS and LC-MS settings. Technological characteristics such as accuracy, precision, complexity, robustness & reproducibility as well as speed and costs determine the choice of method. LC-MS facilitates the reliable, rapid, selective and precise

detection of amino acids in various biological matrices of interest. By being aware of matrix effects and sample preparation steps, LC-MS is useful to even analyze some delicate AAs, their precursors and analogs with reasonable effort as exemplified for arginine, citrulline, canavanine, methionine and homocysteine. Pharmacological intervention strategies to manipulate levels of these AAs are subject to ongoing preclinical and clinical trials for the treatment of a variety of tumors.

Figure Legends

Figure 1

Schematic overview of disciplines developing and applying amino acid detection methodology in research and practice for a broad range of sample matrices

Figure 2

Chemical structures and important characteristics of the three amino acids of interest for metabolic targeting in anti-cancer therapy highlighted in this review.

Figure 3

Illustration of the asparagine-arginine-methionine metabolism network that might be altered in human diseases. This includes the urea cycle (arginine), citric acid cycle (aspartate/asparagine) and the methylation pathway & salvage cycle, all of which are cross-connected through AA intermediates. These pathways potentially reveal novel diagnostic or therapeutic windows for metabolic targeting. AA pathway-related enzymes that are often altered in their function and/or expression in various tumor entities are highlighted in orange (figure adapted from the KEGG PATHWAY database: http://www.genome.jp/kegg/pathway.html?sess=2764b8338258d6286de91bbebe6faf46#metabolism).

Figure 4

AAs of interest for the routine diagnosis and treatment of hereditary metabolic diseases (bottom) and status of AA metabolic targeting in cancer (top)

Note: (i) asparagine was the first targeted amino acid with clinical relevance in cancer; systemic enzymotherapy is in clinical routine for the treatment of acute lymphoblastic leucemia (ALL) and Non-Hodgkin lymphoma (NHL); (ii) arginine deprivation therapy (ADT) is not yet routine but in clinical trial for various (auxotrophic) tumor entities; (iii) methionine has gained clinical importance primarily as PET tracer for diagnostic purposes, i.e. as substitute for [^{18}F]-fluorodeoxyglucose to identify microtumors and metastases in high-glycolytic organs such as the brain; the tracer is in use only at specific clinical sites for glioblastoma multiforme (GBM*) diagnostics and radiotherapy planning; methionine reduction as treatment strategy has been considered via dietary food; enzymotherapeutic approaches are still precarious since methionine as an essential amino acid cannot be completely withdrawn from the human body.

Abbreviations: CA - carcinoma; SCC - squamous cell carcinoma; AML - acute myeloid leukemia; ALL - acute lymphoblastic leukemia; BCAA - branched chain amino acids (Val, Leu, Ile); ArAA = aromatic amino acids (Phe, Tyr, Trp); MSUD - Maple Syrup Urine Disease

Figure 5

Sketch of LC-MS workflow for amino acid detection from sampling to measurement

Note: The addition of internal stable isotope standard(s) is common; non-isotope standards can be considered as optional step.

Figure 6

LC-MS based detection of arginine and its precursors as well as analogs, i.e. citrulline, ornithine and canavanine in a biological matrix (here shown: cell culture supernatant). Citrulline is highlighted as an example for quantification by gating the time frame of retention time and integrating the peak. Peak verification and integration is indicated for both qualifier and quantifier ions of analyte and internal standard as required for the assessment of citrulline concentration in the sample according to the given function.

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Table 1

Table 1: Overview of (A) classical derivatization agents suitable for HPLC, UPLC and LC-MS as well as (B) derivatization agents exclusively developed for use in LC-MS measurements.

(A) "Classical" derivatization agents		
Agent	**Specification**	**Reference**
Ninhydrin	*Ruhemann's purple,* 2,2-dihydroxyindane-1,3-dione	Ruhemann, 1910; Consden et al., 1944; Stein & Moore, 1948; Moore & Stein, 1948
PITC	*Edman's reagent,* phenylisothiocyanate & other isothiocyanates	Edman, 1950; Sherwood et al., 1990; Santa, 2010; Heinriksen & Meredith, 1984; Rydberg et al., 2009
Dabsyl Cl	4-(4-Dimethylaminophenylazo)benzene sulfonyl chloride	Krause et al., 1985; Inoue et al., 2003
Dansyl Cl	5-dimethylamino-1-naphthalenesulfonyl chloride	Maquez et al., 1986; Kang et al., 2006; Baghdady & Schug, 2015
FMOC	9-fluorenylmethyl chloroformate	Bank et al., 1996; Uutela et al., 2009
AQC	6-aminoquinolyl-N-hydrosysuccinimidyl carbamate	Shindo et al., 1997; Badiou et al., 2004; Booger et al., 2008; Salazar et al., 2011
OPA	*o*-phtalaldehyde	Zimmermann, 1930; Klein & Linser, 1932; Zuman, 2004; Alvarez-Coque et al., 1989; Mengerink et al., 2002
(B) Derivatization agents for LC-MS		
Agent	**Specification**	**Reference**
iTRAQ®	isobaric Tags for Relative and Absolute Quantitation, an amine-reactive isobaric tagging agent used in the tagging of peptides and amino acids, two reagents in use: 4-plex and 8-plex, both differ in their fragmentation pattern; only commercial derivatization kit forproteins	Kaspar et al., 2009; Feng, 2011
EASC	10-ethyl-acridone-3-sulfonyl chloride for sensitive UHPLC-MS/MS detection of amino acids with a good chromatographic resolution	Zhao et al., 2015
SPTPP	(5-N-succinimidoxy-5-oxopentyl)triphenyl-phosphonium bromide for the formation of strongproduct ions	Grecoa et al., 2013; Inagaki et al., 2010
TAHS	(p-N,N,N-trimethylammonioanilyl N'-hydroxy-succinimidyl carbamate iodide and its deuterated analogue TAHS-d3 as activated carbamates for sensitive amino acid detection and selected cleavage of the derivatives	Shimbo et al., 2009; Karakawa et al., 2010
APDS	3-aminopyridyl-N-hydroxysuccinimidyl carbamate, synthesized for high-speed analysis in biological fluids	Shimbo et al., 2009b

Table 2

Table 2. Strategies to reduce matrix effects during LC-MS measurements.

Matrix effects: modus operandi*	
Specification	**Reference**
common use of internal, stable isotope standards that are similarly influenced by matrix effects	Nemkov et al., 2015; Van Eeckhaut et al., 2009; Armstrong et al., 2007
standards addition (non-isotope)	Ghassabian et al., 2014
application of multiple lots of biofluids for calibration and assay validation	Matuszewski et al., 2003
clean-up procedures such as solid phase (SPE) or other extractions techniques	How et al., 2014; Cappiello et al., 2008; Armstrong et al., 2007
derivatization (to some extent)	
MS ionization: APCI instead of ESI	Van Eeckhaut et al., 2009; Truffelli et al., 2011; Cappiello et al., 2008
direct electron / electron (impact) ionization	Capiello et al., 2008
use of charged microdroplets	Kulyk et al., 2015

*for review see also : Van Eeckhaut et al,. 2009; Antignac et al., 2005; Matuszewski et al., 2003

Figure 1

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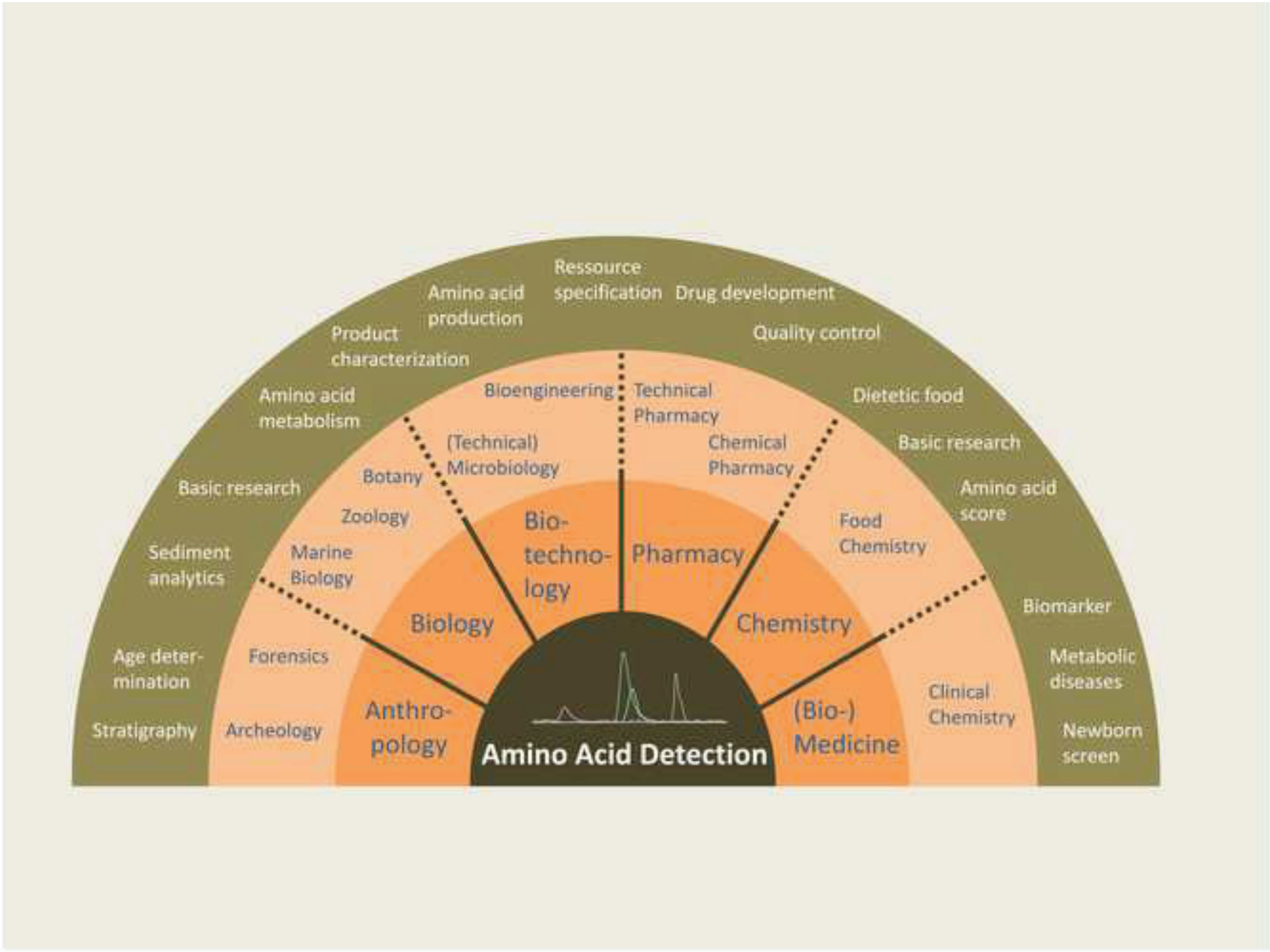

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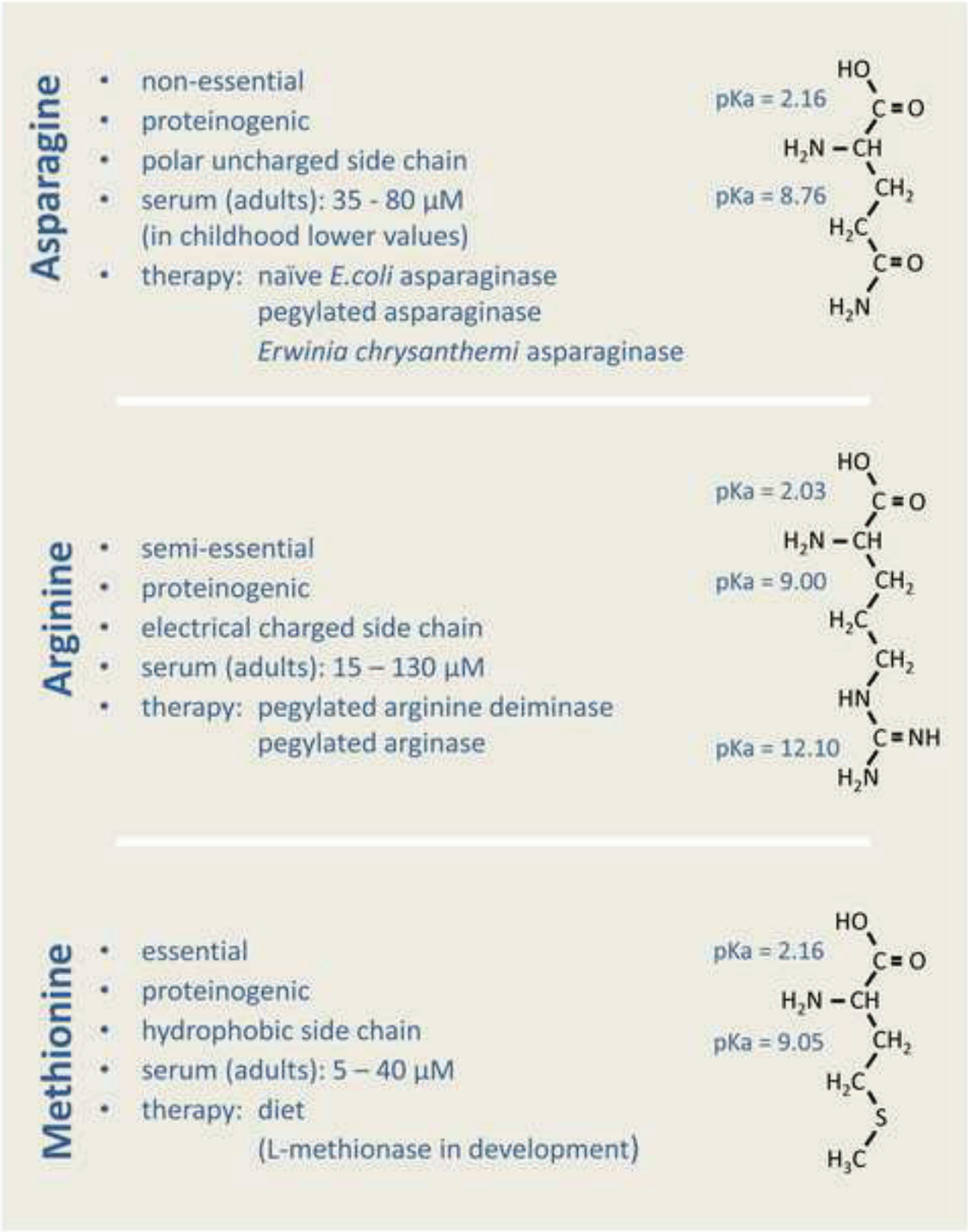

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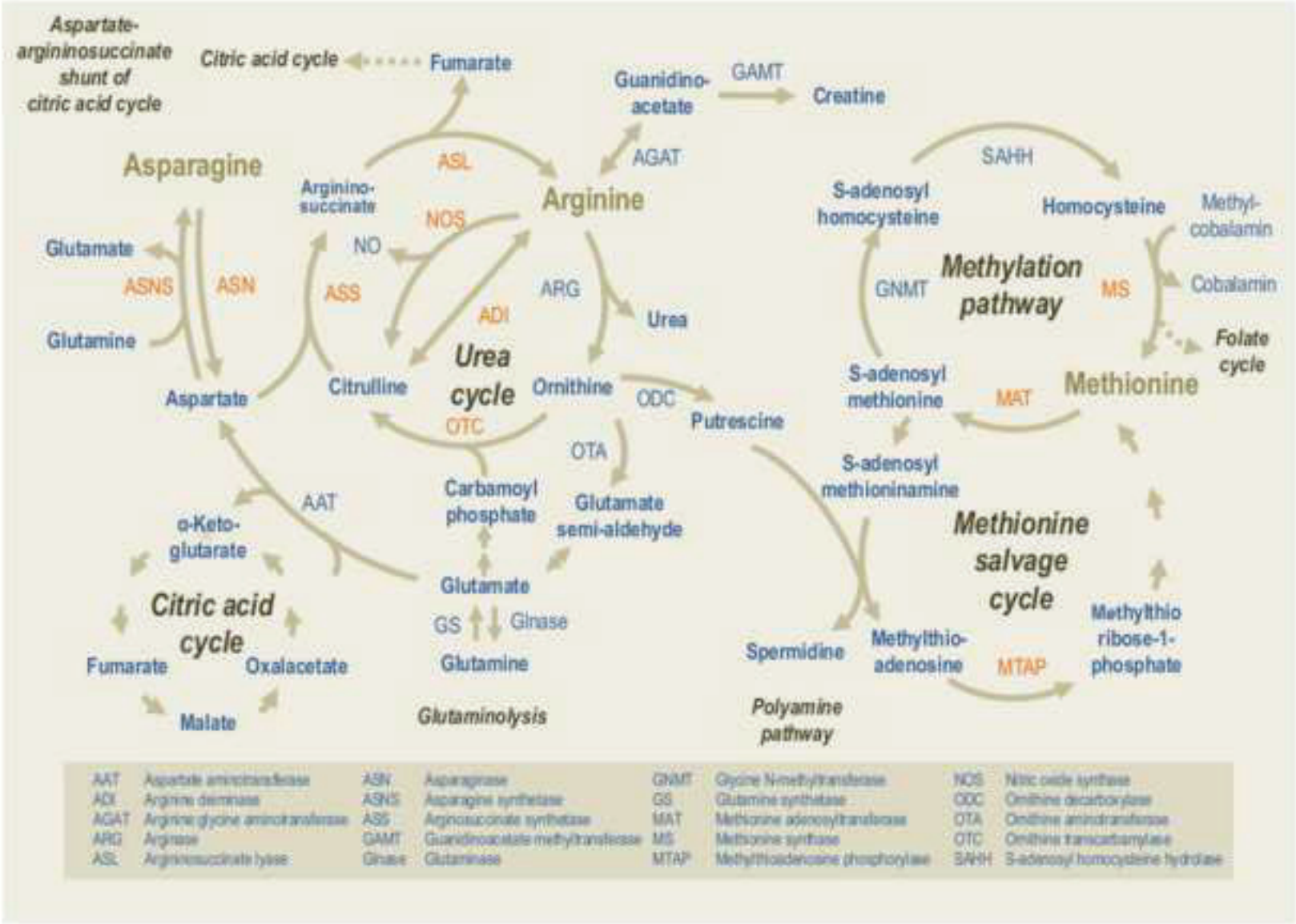

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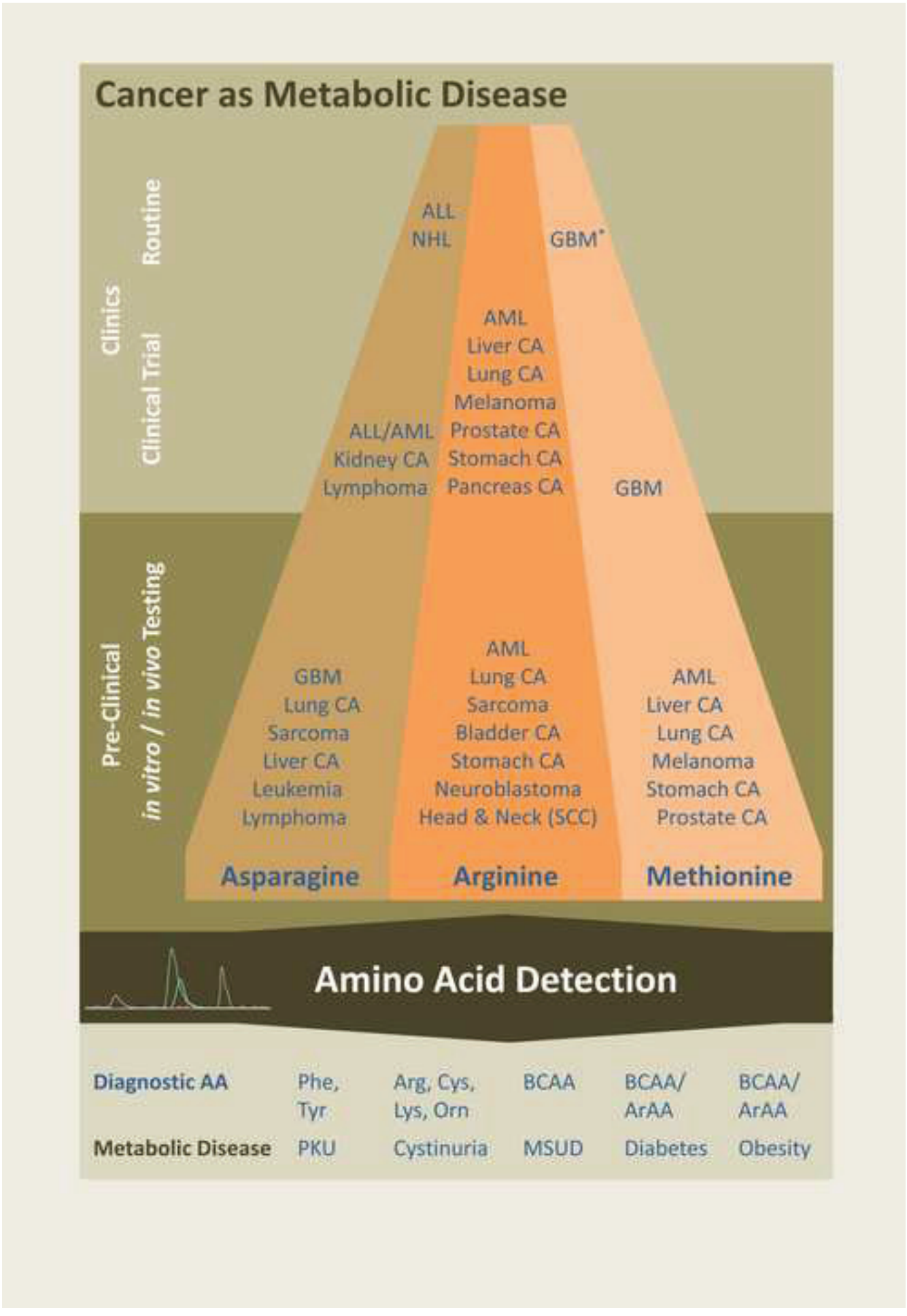

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Figure 6

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