DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Examination of metabolites in human serum, tear as well as wine and wine vinegar samples using mass spectrometry-coupled ultra-performance liquid chromatography

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Abbreviations

AC	Abdominal circumference
ACR	Albumin-to-creatinine ratio
ADC	Arginine decarboxylase
ALDH	Aldehyde dehydrogenase
AMQ	6-aminoquinolone
API	Application programming interface
ApoA1	Apolipoprotein A1
ApoB100	Apolipoprotein B100
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
ASS	Argininosuccinate synthase
AU	Arbitrary unit
AUC	Area under the curve
BCAA	Branched-chain amino acids
BCAT	BCAA transaminase
BCKDHB	Branched-chain ketoacid dehydrogenase
BMI	Body-mass index
CALM	Calmodulin
CAT	Catalase
CAV	Caveolin
CE	Collision energy
CRP	C-reactive protein
DBT	Dihydrolipoamide branched chain transacylase E2
DnsCl	Dansyl chloride
DP	Declustering potential
DR	Diabetic retinopathy
EFSA	European Food Safety Authority
F	Female
FH	Fumarate hydratase
FXN	Frataxin
FDX	Ferredoxin
FMOC-Cl	9-fluorenyl-methyl-chloroformate chloride
GC	Gas chromatography
GFR	Glomerular filtration rate
GLS	Glutaminase
GLUD	Glutamate dehydrogenase
GO	Gene ontology
HbA1c	Glycated hemoglobin
Hcys	Homocysteine
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment for insulin resistance
HPLC	High-pressure liquid chromatography
LC	Liquid chromatography
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
M	Male
MCC	Maximal clique centrality
MDH	Malate dehydrogenase
MQ	Milli-Q
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NAFLD	Non-alcoholic fatty liver disease
NC	Neck circumference
NH	N-hydroxsuccinimide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase
OGDH	Oxoglutarate dehydrogenase
OPA	Ophthalaldehyde
PCA	Principal Component analysis
PDA	Photodiode array
PDK	Pyruvate dehydrogenase kinase
PAO	Polyamine oxidase
RT	Retention time
SD	Standard deviation
SIL	Stable isotope-labelled
SIRT	Sirtuin
SLC	Soluble carrier family
SNX	Sortin nexin
SRM	Selected reaction monitoring
SST	System suitability test
T1D	Type 1 diabetes
T2D	Type 2 diabetes

TCA	Tricarboxylic acid	UV	Ultraviolet
UPLC	Ultra-performance liquid chromatography	WC	Waist circumference
		WHR	Waist-to-hip ratio

1. INTRODUCTION

Metabolomics and proteomics can be considered as state-of the art methods for the examination of the analyte content of complex samples. In the case of both omics methods there are different levels of examinations, such as the shotgun unbiased and targeted analyses. The unbiased methods usually allow for a wider, more comprehensive analysis of the compounds present in the samples but the sensitivity can be compromised and the exact concentration of analytes is hardly achievable. In order to have higher sensitivity, in most of the cases, targeted methods are applied both in proteomics and metabolomics. In these cases, we can not get information on all analytes present in the samples, but the results regarding the target molecules, selected for the analysis have higher sensitivity and absolute quantification is also possible.

Amino acids and biogenic amines constitute a class of compounds with high relevance in examining metabolic changes, as their metabolism is related to the metabolism of carbohydrates, lipids, proteins and nucleotides as well. Having information on the changes related to amino acids and biogenic amines, can provide with a more comprehensive picture, than the examination of each other individual compound type. This fact emphasizes the importance of the analysis of amino acids and biogenic amines in any complex sample.

Diabetes, as one of the so-called population disease, can affect millions of people worldwide. In diabetes the blood glucose level is high and uncontrolled due either to the total absence of insulin, as it can be seen in the case of type 1 diabetes (T1D), or to the insensitivity of the cells to insulin, as it is characteristic to type 2 diabetes (T2D). The presence or absence of insulin makes a great difference and from metabolic point of view there are considerable differences between T1D and T2D. Considering the T2D, its most important risk factors are obesity and prediabetes, and of prediabetes is obesity. It would be important to note the metabolic changes related to the transition of obesity to T2D. The examination of changes in the concentration of amino acids and biogenic amines in T2D and obesity, and their comparison to the controls can provide us useful information on the metabolic conditions.

The concentration of amino acids and of biogenic amines can give important information also in food and beverage analysis. These analytes can be used as quality controls and also to examine the nutraeutical properties of food and beverages. Grapes and grape-derived products can be considered as good models for these type of analyses, as the type of grapes and the process of fermentation greatly influences the amino acid and biogenic amine content of wines and wine vinegars.

2. LITERATURE REVIEW

2.1. Main properties and functions of amino acids

Amino acids are organic compounds characterized by a common structural framework, consisting of a central alpha carbon atom bonded to four distinct chemical groups: a hydrogen atom (H), an amino group (NH_2), a carboxyl group (COOH), and a variable side chain, often represented as 'R' [1]. It is this side chain that distinguishes one amino acid from each other, and it imparts unique chemical and physical properties to each amino acid. The side chains can range from a single hydrogen atom, as in the case of glycine, to complex aromatic rings, as seen in tryptophan. The variations in these side chains dictate the specific chemical behaviors and functions of individual amino acids [2].

Amino acids can be classified into several categories based on their properties. One of the classifications is based on their essentiality. By definition, amino acids that the body cannot synthesize on its own and must be obtained through the diet are called essential amino acids. They include for example phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, and lysine [2,3]. Some other amino acids can be synthesized in the body from other molecules and are not strictly required in the diet, they are the non-essential amino acids, while some others are essential in special cases, these are the conditionally essential amino acids [2–5].

Another classification system categorizes amino acids based on the nature of their side chains. According to this classification amino acids can have non-polar, polar uncharged, acidic, or basic character [1]. Non-polar amino acids, such as leucine and valine, have hydrophobic side chains that repel water, placing them to the hydrophobic cores of globular proteins. Polar uncharged amino acids, like serine and threonine, possess hydrophilic side chains with functional groups that can form hydrogen bonds with water molecules. Acidic amino acids, such as aspartic acid and glutamic acid, carry carboxyl groups in their side chains, contributing to protein charge and function in pH regulation. Basic amino acids, such as lysine feature amino groups in their side chains, rendering them positive charges crucial for ionic interactions in proteins [6].

2.1.1. Amino acids as building blocks of protein

Proteins are fundamental to the structure and function of all living organisms on Earth. Among the more than 800 naturally occurring amino acids [7,8], only 20 of them serve as building blocks of proteins in humans, and their sequence and arrangement determine the protein's structure and function. Those amino acids are called proteinogenic amino acids [3,9].

Proteins are formed by linking proteinogenic amino acids together by a peptide bond and a chain of amino acids linked by peptide bonds is called a polypeptide chain. Proteins are typically composed of one or more polypeptide chains [10].

2.1.2. The physiological functions of amino acids

Amino acids have diverse and multifaceted physiological functions. While their primary function as protein constituents is well-established, these remarkable molecules extend their influence far beyond protein synthesis. Amino acids are involved in various metabolic pathways, gene expression, cell signaling pathways, endocrine functions, antioxidative responses, neurotransmission, and immunity and homeostatic mechanisms that maintain the complexity of life [4,9,11,12]. Along with those functions, amino acids play an important role in carbohydrate metabolism and can be precursors of the tricarboxylic acid (TCA) cycle. Intermediates of the TCA can convert into glucose or acetyl-CoA [13]. In this regard, amino acids can be classified into glucogenic and ketogenic amino acids, or those with both glucogenic and ketogenic features (**Figure 1**).

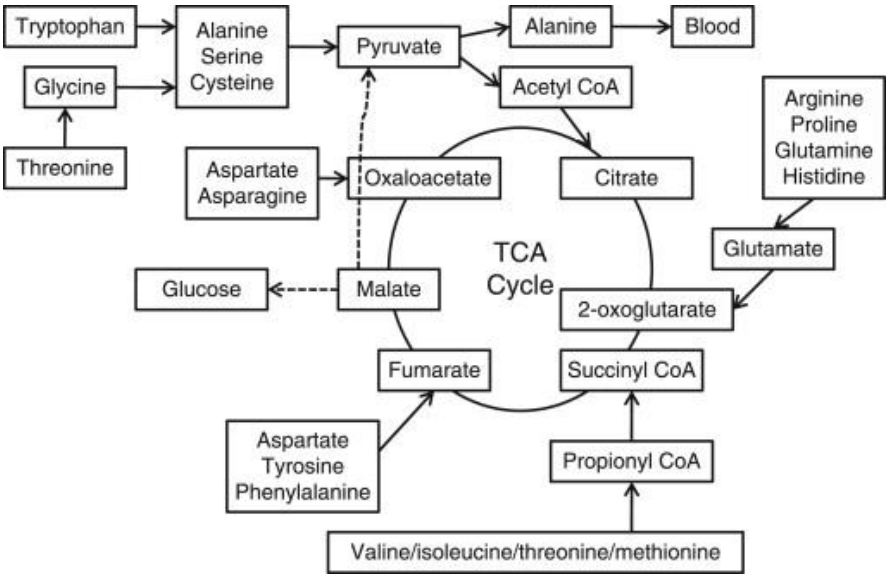


Figure 1. General pathways of amino acids [13].

2.1.3. Amino acids as precursors of biogenic amines

In addition to the main function of amino acids to build up proteins, they act as key precursors to other metabolites, including biogenic amines [9,11].

The metabolic pathway to form biogenic amines from amino acids is the decarboxylation of amino acids by amino acid decarboxylases [14]. The most common biologically active amines are ethylamine (from alanine), agmatine (from arginine), methylamine (from glycine), histamine (from histidine), cadaverine (from lysine), putrescine (from ornithine), phenylethylamine (from phenylalanine), ethanolamine (from serine), tryptamine (from tryptophan), as well as tyramine and dopamine (from tyrosine) which are produced from their precursor amino acids [14–16]. These biogenic amines play important physiological roles in the body; for example, they can act as neurotransmitters, and they can be used as biomarkers and quality indicators [11,17–19].

2.2. Examination of amino acids and biogenic amines

Considering the amino acids as a class of compounds with various physiological properties such as resource of glucose and energy, neurotransmitters, precursors of bioactive molecules as well as building blocks of proteins, it is worth studying their involvement with certain diseases such as T2D and obesity as well as their nutritional value in various food products. In recent years, cutting-edge technologies, such as gas chromatography (GS), nuclear magnetic resonance spectroscopy (NMR), and ultra-performance liquid chromatography (UPLC) combined with mass spectrometry (MS) have seen widespread utilization across various domains of research, encompassing clinical disease diagnostics, biomedical investigations for revealing biomarkers, pharmacological research, as well as food science [20–26].

Separation-based techniques, including liquid chromatography (LC), capillary electrophoresis, and gas chromatography (GC) are the most frequently used methods for the identification and quantification of various compounds, including amino acids and biogenic amines in complex samples [27,28].

To date, UPLC, a modified high-pressure liquid chromatography (HPLC) is the most widely used technique because of its great versatility [29,30]. A decrease in the particle size of stationary phase provides higher resolution and efficiency in a shorter time compared to the conventional HPLC, but higher pressure is required [30].

At present, UPLC is the preferred method for the analysis of amino acids and biogenic amines in diverse samples without derivatization or utilizing either manual or automated precolumn or postcolumn derivatization procedures with various derivatizing agents [31–34]. Mass spectrometry can be used as a supplementary tool for UPLC in order to increase the selectivity and sensitivity of the analyses as well as for the confirmation of the analytes identified with ultraviolet (UV) or fluorescent detection [35,36].

2.2.1. Derivatization methods for enhanced detection

Amino acid analysis by LC and optical detection can be improved by additional sample preparation and derivatization which leads to enhanced sensitivity and selectivity [37].

Derivatization generally involves a chemical reaction between a target analyte and a reagent to change the chemical and physical properties of the target analyte. With these modifications of the analytes, the derivatization improves detectability, separation, and volatility and stabilizes the analytes enabling better chromatography [27,33,37–40].

There are several derivatization methods using dansyl chloride (DnsC1), ophthalaldehyde (OPA), 9-fluorenylmethylchloroformate chloride (FMOC-Cl), or AccQ-Tag [31–34].

The AccQ-Tag derivatization has been widely used in amino acid and biogenic amines analysis due to its advantages including simple and fast derivatization reaction of amino groups, making the derivatives more stable and detectable by both UV and fluorescence [25,34,41,42].

2.2.2. Chemistry of AccQ-Tag derivatization

The AccQ-Tag method is a pre-column derivatization technique. It uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag Ultra, AQC) reagent for derivatization reacting with both primary and secondary amines. At first, the primary and secondary amino groups in the complex sample react with the AQC reagent (**Figure 2**). Secondly, the excess AQC reagent reacts with water to form 3 byproducts: 6-aminoquinolone (AMQ), N-hydroxsuccinimide, and CO_2. Finally, the major byproduct AMQ and the AQC excess react with each other and form the highly stable bis-aminoquinoline urea, which shows a derivatization peak on the chromatogram. These byproducts do not affect the identification or the quantification of the amino acids [43].

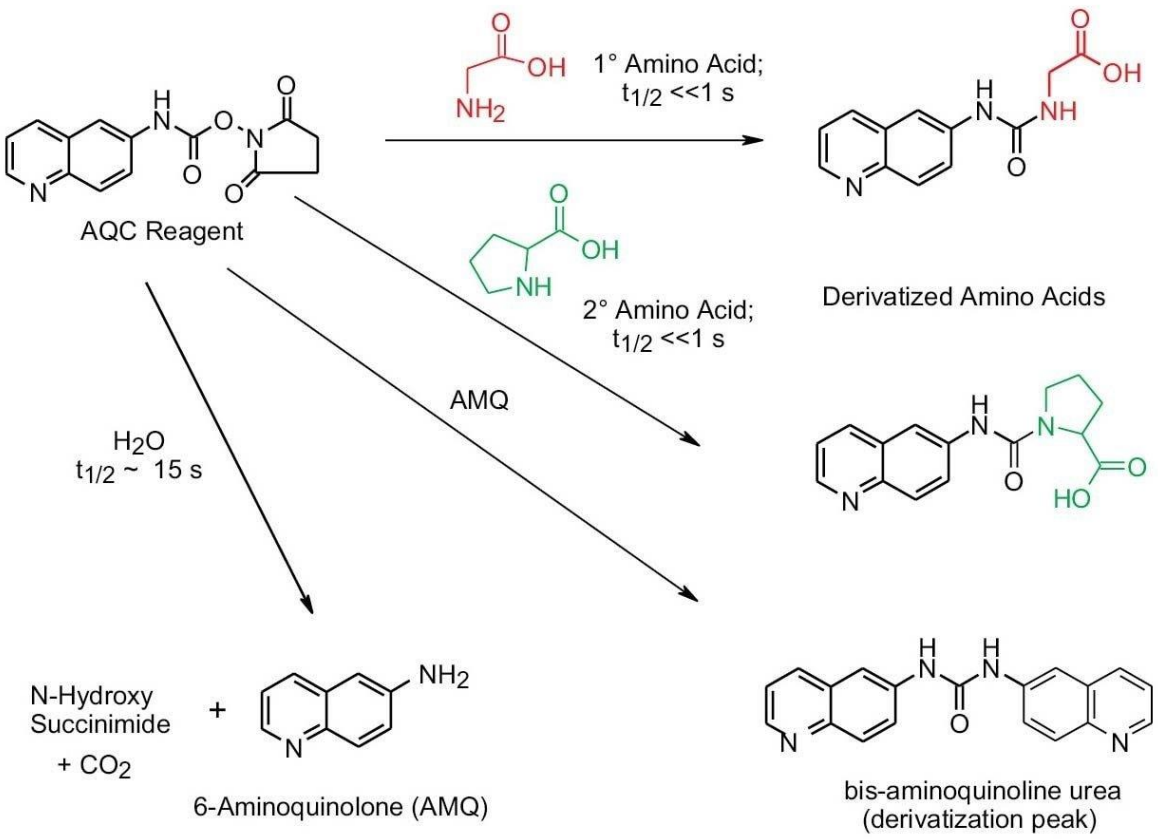


Figure 2. Chemistry of the AccQ-Tag *Ultra reagent (AQC) derivatization of primary and secondary amino acids* [44].

As a chemical reaction, derivatization requires an optimal pH medium. Mixing the sample with borate buffer allows the optimal pH for the AccQ-Tag derivatization reaction. In this optimal environment, AccQ-Tag reagent can completely derivatize all amino acids and amino group containing analytes in the sample, therefore, it will react besides amino acids with biogenic amines as well [38,40,45].

The main advantages of utilizing AccQ-Tag derivatization are the fast derivatization, low sample consumption as well as significantly increased detection sensitivity and resolution [46].

2.3. Amino acids and diseases

Recent studies have found that the amino acids can be used as potential biomarkers for myasthenia gravis [47], some forms of cancer [48], non-alcoholic fatty liver disease (NAFLD) [49], etc. In addition, amino acids may be associated with the development of metabolic disorders, insulin resistance, and T2D [50–53].

Diabetes is among the most prevalent metabolic disorders. Although there are currently 540 million people living with diabetes worldwide according to the report of the International

Diabetes Federation, this number is expected to increase to 643 million within ten years and 783 million by 2045, as they predicted in 2021 [54]. Moreover, only 10% of individuals who are suffering from diabetes were diagnosed with T1D while the remaining 90% have T2D [54]. Extensive investigations have provided compelling evidence that obesity stands as an important risk factor driving the onset of T2D [55–58]. The likelihood of developing T2D escalates significantly, with obese individuals exhibiting a sevenfold increase in risk and overweight individuals presenting a threefold elevation in risk of developing T2D [59,60]. A substantial proportion of individuals diagnosed with T2D also concurrently present with overweight or obesity, often accompanied by central visceral adiposity, underscoring the fundamental significance of adipose tissue and obesity in the pathogenesis of T2D [61].

When considering the underlying mechanisms of obesity-induced T2D, insulin resistance and dysfunction of pancreatic beta cells were found to be important. The insufficient reaction to insulin, even when insulin levels are in normal or elevated range defines insulin resistance [62]. Consequently, this lack of effectiveness of insulin leads to hyperglycemia [63–65]. Obesity results in an elevation of glucose levels in the bloodstream, causing an increase in insulin production by beta cells as a compensatory response to hyperglycemia. However, with time, beta cells' functions become impaired, leading to a decline in insulin secretion [56,60].

Since T2D is typically defined by the presence of hyperglycemia, its diagnosis often involves assessment of the level of blood glucose in the first line [66,67]. However, relying solely on glucose monitoring may not provide a comprehensive understanding of the biochemical aspects of this condition.

Over the years, extensive researches on carbohydrate, protein, and lipid metabolism [68,69] have made significant advances in uncovering the roles of these compounds in the development of T2D and obesity. In addition, metabolomics studies have been carried out in order to investigate the significance of amino acids, these studies offer valuable insights into cellular functions that play critical roles in the pathophysiology of T2D [66,70–73].

Amino acids emerged as the predominant metabolites in distinguishing between individuals who are healthy and those who are obese or diabetic. This distinction is primarily attributed to their strong correlations with critical indicators of metabolic disorders, including the homeostatic model assessment for insulin resistance (HOMA-IR) and glycated hemoglobin (HbA1c) [74]. Furthermore, serum amino acids have demonstrated notable associations with the risk factors for T2D and obesity [75–77].

In response to the needs of earlier diagnosis, better predicting the prognosis, prevention of complications, and cost-effective treatments of obesity and diabetes, researchers conducted

numerous studies on proteome and metabolome profiling in various biological fluids in obesity or T2D. These studies were carried out on blood serum or plasma, saliva, tears, and urine collected from donors with such conditions [66,68,78–82].

It is recognized that the chronic metabolic alterations associated with T2D can give rise to a range of complications which are typically categorized as macrovascular and microvascular [83]. Diabetic retinopathy (DR) is amongst the most prevalent microvascular T2D-induced complications of the eyes, furthermore, it can lead to vision impairment and blindness [84]. Several tear proteins, including 2-microglobulin, apolipoprotein A-1, heat shock protein 27, immunoglobulin lambda chain, lactotransferrin, lipocalin 1, lipophilin A, lysozyme C, and nerve growth factor were identified as potential biomarkers for the DR [81,85–87].

Tear fluid may be an ideal source for biomarker discovery concerning diabetic retinopathy due to its unique composition, accessible collection, closeness to the disease location, and minimal cell contamination [88,89]. Since the sample collection method is non-invasive for patients, and easy to handle for health service providers [90], tears can be an alternative sample choice for metabolite analysis in diabetic patients.

Branched-chain and aromatic amino acids as well as glycine are already known as biomarkers for obesity and T2D [76,91–93]. However, their presence in the tears of patients has not been studied as widely as in serum or other types of samples.

2.4. Biogenic amines and diseases

Many researchers examined biogenic amines in order to identify them as potential biomarkers for various diseases. For example, putrescine was found as a potential biomarker for breast cancer in serum, but not in urine [19]. The concentration of putrescine and cadaverine was significantly elevated in cancer tissue compared to the unaffected tissue, while serum putrescine but not cadaverine was higher, in patients with cancer compared with healthy controls [94]. Another study found an increased level of putrescine and a decrease in spermine in plasma samples of patients with chronic renal failure [95]. For other biologically active biogenic amines, there is still a gap regarding their involvement in the development of metabolic disorders. The levels of putrescine, spermine, and spermidine, also known as polyamines, were studied in human serum samples collected from patients with obesity and found no significant differences in their concentration between male and female patients [96]. According to another study, the serum level of putrescine was higher in patients with T2D as compared to non-diabetic patients, while the levels of ornithine and arginine were significantly

lower [97], and the elevated level of putrescine correlated with the HbA1c level [98]. In general, a higher level of putrescine in patients with T2D and obesity was observed compared to healthy subjects. This phenomenon indicates an upregulation of the putrescine biosynthesis pathway, specifically involving the production of putrescine from ornithine through the biosynthetic enzyme ornithine decarboxylase (ODC), as well as the degradation of spermine by polyamine oxidase (PAO), and the uptake of these components [98]. Various amines, including kynurenine, spermidine, and creatinine have been linked to the transition of gestational diabetes to T2D [99], and the urinary tyramine level was lower in patients with metabolic syndrome than in control subjects [100]. Another biogenic amine, methylamine which is present commonly in higher amounts in food products was shown to stimulate glucose uptake of adipocytes in a cell culture study [101].

2.5. Amino acids in grape-derived products

Grapes are the major raw material in the production of wines and can also be consumed in the forms of grape juice, must, and vinegar as drinks.

Wine is the result of alcoholic fermentation of grape must by *Saccharomyces* species. Besides wine, another product, which can be produced from the grapes is vinegar, specifically wine vinegar. This type of vinegar is essentially an acetic acid solution obtained through the *Acetobacter sp.* bacterial fermentation of the grape must [102].

In the field of the study of wine, also known as enology, extensive research works on amino acids and biogenic amines are carried out aiming to explore their health benefits, nutritional value, toxic effects, hygiene issues, and wine quality [18,103–108]. The study of the wine and wine vinegar composition provides valuable insights into their potential use as functional food. The amino acids in wine and wine vinegar can play a crucial role as a nitrogen resource during the fermentation process, favoring the growth of lactic acid bacteria and yeast [109]. Amino acids account for approximately 30-40% of the overall nitrogen in wine [110,111] and their profile may serve as a useful indicator of wine quality. Besides that, amino acids contribute to the aroma, flavor, and overall characteristics of wines [112,113].

Wine and wine vinegar have been the subject of numerous scientific studies in terms of their content, however, the results are not consistent all the time due to the application of different instrumentations, analytical methods, and tools.

The most common amino acids found in wine are proline, glutamate, and arginine according to some studies [114–116]. Wong *et al.* [113] suggest that the presence of proline,

alanine, aspartate, phenylalanine, and threonine in high concentrations can potentially influence the flavor and color of wine. According to other studies, the amino acid and biogenic amine content of wine is highly dependent on the variety of grapes, vintage (year of collection), and the microbes used for fermentation [117–119].

The Tokaj wine region, a UNESCO World Heritage Area since 2002, is renowned for its exceptional, highly-qualified wines. Furmint is one of the grape varieties frequently used in winemaking in this region yielding a diverse range of sweet and dry wines [118]. Aszú wine is produced from the Furmint wine poured over the grapes infected with *Botrytis cinerea.* As a result of the *Botrytis* infection, called noble rotting, the sugar content of the berries is increased and a unique taste is formed [120–122]. Essence, a refined Aszú wine with high sugar concentration and a consistency similar to bee honey can also be made by winemakers in this region [122]. In order to produce the Essence, the botrytized berries are not pressed, instead, the grape juice is produced by the effect of internal pressure of the botrytized berries. This juice undergoes a gradual and spontaneous fermentation process over time [120,121,123].

2.6. Importance of biogenic amines in the grape-derived products

Biogenic amines are produced from their precursor amino acids by the decarboxylase enzyme of the microorganisms during the fermentation of beverages [108] or can be present in the grape or raw material itself [114,118,124–126].

The presence and concentration of amino acids and biogenic amines, as well as of other nutritional compounds in grape-derived products are mainly determined by grape variety, weather, and the manufacturing processes [111,127,128]. Before or during the fermentation, the wine components may come in contact with different microbial agents such as *Botrytis cinerea*, essential for the noble rotting of grapes [120–122], *Saccharomyces cerevisiae*, required for alcoholic fermentation [129] and *Acetobacter sp*. utilized in vinegar production [102].

Biogenic amines in wine and other grape-derived products are mostly related to toxicity, quality and hygienic issues [130–133]. Putrescine and cadaverine can indicate problems related to food processing or storage and can have toxic effects in higher concentrations, while histamine can be of concerns for those having problem with its degradation [130,134,135].

3. AIMS AND OBJECTIVES

Amino acids and biogenic amines as molecules related to the metabolism of all big classes of compounds such as carbohydrates, lipids, proteins and nucleotides, can be considered as analytes, whose concentration changes can provide a snapshot of metabolic conditions present in the complex samples. Having available an analytical method developed in our laboratory for the determination of the concentration of 20 human proteinogenic amino acids, 3 selected amino acids and of 10 biogenic amines we aimed to utilize this methodology for gaining metabolic snapshots in two, totally different models, reflecting two unrelated metabolic conditions.

In our first study, our aim was the profiling of amino acids and biogenic amines in the serum and tear samples obtained from patients with T2D, patients with obesity, and sex- and age-matched controls. Knowing that amino acids and some biogenic amines are deeply involved in the pathophysiology of obesity and T2D, we aimed to conduct a complex data analysis, to examine the altered metabolic pathways and networks to get a new perspective in examining metabolomics data and understanding the pathophysiological mechanism driving obesity and T2D.

In our second study, our aim was to examine the amino acid and biogenic amine composition of some grape-derived beverages. The quality control and also assessing the nutraceutical properties of food and beverages has extremely high importance. As far as the grape variety and the type of fermentation both influence the amino acid and biogenic amine content of wines and wine vinegars we aimed to test our methodology on our selected complex model system. We aimed to determine the amino acid and biogenic amine concentration of Furmint wine, the botrytized Aszú wine, Essence, and wine vinegars originating from the Tokaj region. We also aimed to compare Furmint-Aszú pairs originating from the same winery and year, in order to observe the effect of the presence of botrytized grapes on wine amino acid and biogenic amine content.

With these two different studies we aimed to demonstrate the utility of the developed analytical methodology for getting useful information both on pathophysiological changes related to disease and on the effect of grape type, variety and fermentation conditions of wine and wine vinegars.

4. MATERIALS AND METHODS

4.1. Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MI, USA) if not stated otherwise. A standard mixture of 17 amino acids, including alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine, along with the AccQ-Tag Ultra derivatization kit and AccQ-Tag Ultra eluent A and B were purchased from Waters (Milford, MA, USA). Tryptophan was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

4.2. Study materials

In our study, we examined the amino acid and biogenic amine content in human serum and tears as well as in grape-derived products, including white wines, essence, and wine vinegar.

4.2.1. Recruited study subjects

85 subjects were recruited for this study, including 26 patients with T2D, 31 subjects with obesity, and 28 healthy volunteers. The study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent, and the study was approved by the Ethics Committee of the University of Debrecen (4845B-2017). The study groups were matched for age and sex. The patients in the T2D group had an average age of 54 years with a 1:1 ratio of male to female. Similarly, in the obese group, the mean age was 53 years with a 1:1 male-to-female ratio. In the healthy group, the average age was 55 years, and the gender ratio of male to female was 1:1. The grouping of the patients were done by specialist in internal medicine according to the available medical guidelines. The main difference between the obese and T2D groups were their blood glucose and HbA1c levels. The presence of type 1 diabetes or other, non T2D-related diseases was among the exclusion criteria. The donors recruited into the T2D group were patients of the Internal Medicine Clinique of the University of Debrecen.

4.2.2. Sample collection

Fasting blood samples were collected from all participants (n=85) in native vacuum tubes, centrifuged for 15 minutes at 2000 x *g* and the supernatant (serum) was collected. Sera were aliquoted and stored at -70°C until they were processed and analyzed.

Basal tear samples from forty of the 85 participants were collected using a glass capillary [90] by professionals. Afterwards, the samples were centrifuged for 15 minutes at 2000 x *g* and the supernatant was collected and stored at -70^{0}C until further examination. The donors were examined by ophthalmologists for the presence of ocular diseases and diabetic retinopathy. Tear samples could be collected from 19 patients with obesity and 21 patients with T2D. 11 patients with T2D had no diabetic retinopathy and 10 had some form of DR (9 with non-proliferative and 1 with proliferative stage).

4.2.3. Grape-derived products

Four types of grape-derived beverages, including Aszú wine (n = 8), Furmint wine (n = 8), Essence (n = 2), and wine vinegar (n = 4) from the Hungarian Tokaj region, a UNESCO World Heritage site, were selected for examination. The samples were from different wineries and years as indicated in **Table 1**.

Table 1. Details of grape-derived products.

Sample ID	Type	Year	Winery	Additional data
5A	Aszú	2000	Köhler	5 baskets
62A	Aszú	2008	Szepsy	6 baskets
60A	Aszú	2013	Szepsy	6 baskets
4A	Aszú	2017	Szepsy	Újhegy
337A	Aszú	2013	Szent Tamás	6 baskets
40A	Aszú	2013	Sauska	6 baskets
39A	Aszú	2013	Bardon	-
2134A	Aszú	1940	Muscal	6 baskets
63F	Furmint	2008	Szepsy	-
61F	Furmint	2013	Szepsy	Hasznos
334F	Furmint	2017	Szepsy	-
338F	Furmint	2013	Szent Tamás	-
44F	Furmint	2013	Sauska	Birsalmás, dry
45F	Furmint	2013	Bardon	Alpha, dry

87F	Furmint	2016	Szepsy	-
339F	Furmint	2017	Patricius	-
2173E	Essence	1940	Muscal	-
46E	Essence	2013	Pauleczki	Szőlőbirtok muscat lunel
EC1	Grape vinegar	-	-	late harvest, starting from concentrated grape must
EC2	Aszú balsamic vinegar	-	-	furmint wine vinegar ripened on aszú pomace bed
EC3	Tokaj noble vinegar	-	-	ripened on pomace bed of aszú berries for 5 years
EC4	Furmint vinegar	-	-	-

4.3. Sample processing for the analysis

In order to eliminate macromolecules and other contaminants from all samples, we performed a filtration using Nanosep 3 kDa spin columns (Pall Corp, New York, NY, USA) as follows:

100 µl of thawed serum sample was filtered using a Nanosep 3kDa spin column at 12,800 x g, at 4°C for 10 minutes and the flow-through was used for the analysis.

In case of tear samples, 3 µl of thawed tear was diluted with Milli-Q (MQ) water to 50 µl final volume and filtered similarly to the serum samples. The filtrated sample was completely dried in a vacuum centrifuge (ThermoScientific, San Jose, CA, USA) prior to the AccQ-Tag derivatization process.

200 µl of the sample was transferred to a Nanosep 3kDa spin column in the case of each beverage which were then centrifuged at 12,800 x g at 4°C, for 10 minutes, by repeating the cycle 3 times. The flow-through was completely dried in a vacuum concentrator (ThermoScientific, San Jose, CA, USA) before the derivatization.

4.3.1. Derivatization of amino acids and biogenic amines in the samples

Amine-group containing compounds in the samples were derivatized with AccQ-Tag Ultra Derivatization kit according to the manufacturer's protocol (Waters, Milford, MA, USA). For the derivatization of serum samples, 10 µl of filtered serum was mixed with 70 µl of AccQ-Tag Ultra borate buffer, and 20 µl of AccQ-Tag Ultra reagent. For the derivatization of tear

samples, the dried tears were resuspended in 80 µl AccQ-Tag Ultra borate buffer and 20 µl of AccQ-Tag Ultra reagent was added to the samples. In the case of wine and other grape-derived samples, 70 µl of AccQ-Tag Ultra borate buffer was added to the dried samples first. After redissolving samples completely, the pH was adjusted to ≥8 using 2 M NaOH, and then the volume was adjusted to 80 µl with borate buffer. Finally, the sample was subjected to derivatization by adding 20 µl of AccQ-Tag Ultra reagent.

A stock solution of analytes (2500 pmol/µl for each amino acid and 1250 pmol/µl for each biogenic amine) was prepared and kept at -20°C until the analysis. The stock solution was stable for 3 months in the freezer. Before the analysis, a serial dilution of the stock solution was performed to achieve the concentration series used for the calibration curve. 20 µl of each calibration standard was mixed with 60 µl of AccQ-Tag Ultra borate buffer and 20 µl of AccQ-Tag derivatization reagent in glass vials for derivatization reaction.

In addition, gradient blank, reagent blank, and System Suitability Test (SST) solutions were prepared. The gradient blank was a mixture of borate buffer and MQ water for testing the quality of the mobile phase and solvents used for the sample preparation. The reagent blank contained borate buffer, AccQ-Tag Ultra derivatization reagent, and MQ water dedicated to testing the quality of the derivatization reagent. SST solution was composed of borate buffer, AccQ-Tag Ultra derivatization reagent, and a working solution, which was prepared from the stock solution diluted to 30 pmol/uL for each amino acid.

After adding the derivatizing reagent to all types of samples, calibration standards, gradient blank, reagent blank, and SST, an incubation was carried out at 55°C for 10 minutes. After cooling down, the derivatized samples were analyzed by an Acquity H-class UPLC system (Waters, Milford, MA, USA) coupled to 5500 QTRAP (Sciex, Framingham, MA, USA) mass spectrometer.

4.4. UPLC-MS/MS analysis

LC separation of the components was performed on the Acquity H-Class UPLC system equipped with an UV detector. The separation was done on an AccQ-Tag Ultra C18 column (1.7 µm; 2.1 × 100 mm, Waters, Milford, MA, USA) guarded by an Acquity in-line filter (0.2 µm; 2.1 mm, Waters, Milford, MA, USA) column.

In the mobile phase, solvent A was 100% AccQ-Tag Ultra eluent A; solvent B was composed of 10% AccQ-Tag Ultra eluent B dissolved in LC–MS grade water; solvent C was

LC-MS grade water; and solvent D was 100% AccQ-Tag Ultra eluent B. An in-house developed 11-minute gradient was used according to **Table 2**.

Table 2. Gradient parameter values for LC method.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Mobile phase D (%)	Curve
0.00	10.00	0.00	90.00	0.00	initial
0.29	9.90	0.00	90.10	0.00	6
3.50	9.90	0.00	90.10	0.00	6
4.60	9.90	25.00	65.10	0.00	7
5.49	9.00	80.00	11.00	0.00	6
7.10	8.00	25.00	57.90	9.10	6
7.30	8.00	15.60	57.90	18.50	6
7.50	8.00	12.00	57.90	22.10	6
8.20	7.80	0.00	77.20	15.00	6
8.30	4.00	0.00	36.30	59.70	6
8.55	4.00	0.00	36.30	59.70	6
8.60	4.00	65.00	26.00	5.00	6
9.20	4.00	60.00	36.00	0.00	6
9.70	10.00	0.00	90.00	0.00	6
10.90	10.00	0.00	90.00	0.00	6

The flow rate was 0.650 ml/min, the column temperature was set to 54°C, and the samples were kept at 4°C in the autosampler. The PDA detector of the instrument was set to 260 nm wavelength. The chromatograms were integrated by the Empower 3 software (Waters, Milford, MA, USA).

Selected/Multiple Reaction Monitoring (SRM/MRM)-based targeted mass spectrometry analyses were carried out on the 5500-QTRAP (Sciex, Framingham, MA, USA) mass spectrometer, controlled by the Analyst software (version 1.6.3., Sciex, Framingham, MA, USA). The samples were ionized by using electrospray ionization with 5500 V capillary voltage and the positive ion mode SRM spectra were recorded. The other acquisition parameters were set as follows: the ion source gas 1 was 30 psi, the ion source gas 2 was 50 psi, the curtain gas

was set to 30 psi, and the source temperature was 500°C. The SRM/MRM transitions and the collision energies applied for each analyte are shown in **Table 3**.

Table 3. SRM/MRM parameters applied for the examination of the analytes.

Analyte	RT window (min.)	Q1 (*m/z*)	Q3 (m/z)	DP (eV)	CE (eV)
Histidine	1.06-1.90	326.00	171.00	230	18
Asparagine	1.30-2.14	303.00	171.00	150	15
Taurine	1.51-2.35	296.15	171.00	120	25
Serine	2.00-2.84	276.00	171.00	230	18
Glutamine	2.16-2.00	317.00	171.00	210	16
Arginine	2.28-3.12	345.00	171.00	110	21
Histamine	2.39-3.23	282.15	171.00	120	25
Glycine	2.54-3.38	246.00	171.00	230	15
Ethanolamine	2.84-3.68	232.08	171.00	120	25
Aspartate	3.10-3.94	304.00	171.00	160	16
Methylamine	3.48-4.32	202.00	171.00	120	25
Glutamate	4.45-5.29	318.00	171.00	210	16
Citrulline	4.54-5.38	346.20	171.00	120	25
Threonine	5.12-5.96	290.00	171.00	120	10
Alanine	5.58-6.42	260.00	171.00	160	13
Ethylamine	5.77-6.61	216.00	171.00	120	25
Proline	6.01-6.85	286.00	171.00	130	15
Ornithine	6.64-7.48	473.30	171.00	120	25
Cysteine	7.06-7.90	291.00	171.00	120	13
Lysine	7.25-8.09	487.00	171.00	230	22
Tyrosine	7.30-8.14	352.00	171.00	210	15
Putrescine	7.38-8.22	429.15	171.00	120	25
Methionine	7.52-8.36	320.00	171.00	120	16
Serotonin	7.58-8.42	347.20	171.00	120	25
Valine	7.63-8.47	288.00	171.00	100	16
Cadaverine	7.65-8.49	443.20	171.00	120	25

Tyramine	7.68-8.52	308.20	171.00	120	25
Isoleucine	8.08-8.92	302.00	171.00	120	25
Leucine	8.08-8.92	302.00	171.00	120	25
Serotonin	8.14-8.98	517.20	171.00	130	15
Phenylalanine	8.30-9.14	336.00	171.00	160	20
Tryptophan	8.41-9.25	375.00	171.00	160	20
SIL Tryptophan	8.41-9.25	388.00	171.00	120	25
Tryptamine	8.54-9.38	331.20	171.00	120	25
Phenylethylamine	8.54-9.38	292.20	171.00	230	18

Q1 *m/z* – parent ion, Q3 *m/z* – fragment ion, RT – retention time, DP – declustering potential, CE – collision energy, SIL- stable isotope-labelled [38].

During the analysis, one microliter of the sample was injected, and two technical replicates were recorded. A 10-point calibration curve (0.25-30.0 micromol/L range) was prepared and used to determine the concentration of analytes. The recorded UV chromatograms were analyzed with the Empower 3 software, and the SRM/MRM spectra were analyzed with Skyline (v.20.2, www.maccosslab.org, downloaded on 21 January 2022) software. The analytes were identified based on their retention time and verified using the SRM/MRM transitions. Where possible, the UPLC data were used for quantification. In the case of analytes with lower concentrations than the detection limit of the UPLC, the mass spectrometry data exported to the Skyline were used for quantification. The area under the curve (AUC) was extracted in the case of each analyte and used for further examinations.

4.5. Statistical analysis

The determination of the statistically significant differences among the groups one-way ANOVA analysis was applied. After running post hoc Tukey's test, only the results with an FDR < 0.05 were further considered. For correlation analysis, we applied non-parametric Spearman correlation tests to study the associations between the analytes and the other parameters. FDR correction was applied and results with FDR < 0.05 were kept.

In the case of beverage analysis, first a descriptive statistics was carried out, followed by the Mann-Whitney test to analyze the mean value, range, and standard deviation (SD). For statistical analyses, the Graph Pad Prism software (version 8.0.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) was used. The p-value of $p \leq 0.05$

was considered statistically significant. Principal component analysis (PCA) was computed by the prcomp function of the R stats package. The first three principal components were extracted, and the 3D plot was generated utilizing the plotly library. The heatmap was created by the srplot of the R stats package.

4.6. Network analysis

Enzymes having a role in the degradation or synthesis of the analytes with statistically significant change between the groups were retrieved from the MetaCyc (MetaCyc.org) [136], as part of the BioCyc (BioCyc.org) database collection via MetaCyc's application programming interface (API) with the brendaDb R package (v1.6.0) [137]. We used R (v4.0.3) for table operations and reorganization of the downloaded data [138]. The enzyme dataset was complemented with the relevant amino acid transporters based on a comprehensive review article [139]. The whole dataset including a list of the above-mentioned enzymes and transporters was queried on the STRING database (v11.5) [140] in order to generate the protein-protein interaction networks. From the STRING, we acquired networks containing the query proteins along with their up to 50 first-shell interactors. The confidence level was set to very strict, 0.9. The general workflow of the network analysis is shown in **Figure 3**.

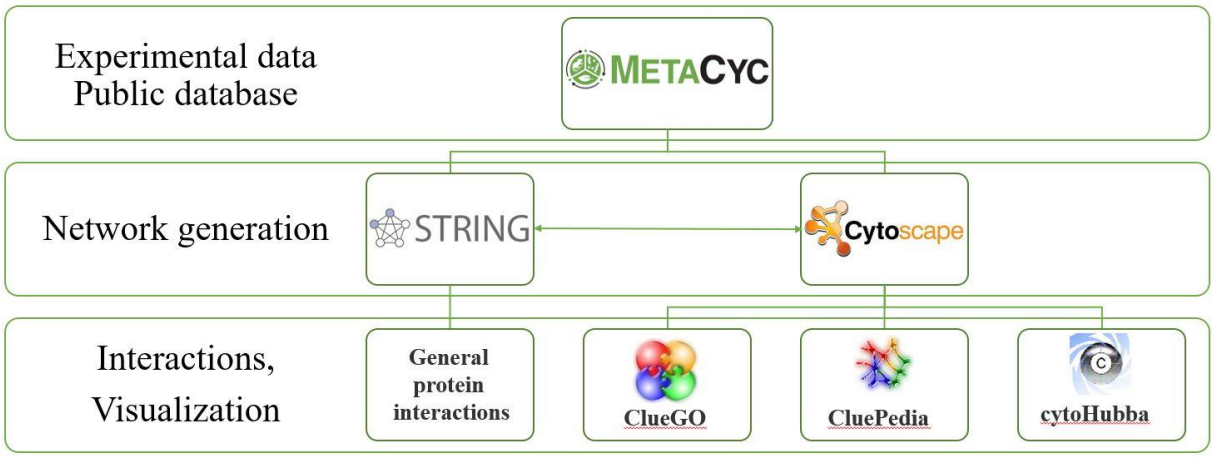


Figure 3. Network analysis workflow.

The enzymes and transporters of amino acids and their first shell interactors which are closely involved in obesity and T2D were imported from the STRING to the Cytoscape software v3.9.0 [141]. On the Cytoscape, the pathway analysis was performed using its ClueGO v2.5.8 plug-in [142], and the parameters were set to p-value ≤ 0.05. and all proteins were extensively searched using the gene ontology (GO) biological pathways database with a

threshold of 1000 genes. Next, we processed the same data using CluePedia v1.5.8 [143] in order to examine the activation, inhibition, catalysis, binding, and co-expression of the proteins or enzymes. The CluePedia-generated interaction networks were further investigated on the CytoHubba v0.1 [144] to determine the top hub proteins in the network based on Maximal Clique Centrality (MCC). In return, these top hub proteins were investigated to determine their interactions with the help of CluePedia.

5. RESULTS

Using our developed method for the analysis of the amino acids and biogenic amines we carried out two studies and we examined amino acids and biogenic amines in human samples, including serum and tear in the healthy conditions and pathological states as well as in grape-derived products, respectively.

5.1. Examination of amino acids and biogenic amines in human samples

In this study, we examined the concentration of 23 amino acids and of 10 biogenic amines in human samples collected from healthy individuals and patients with obesity or with T2D. In addition, we studied the correlation of the concentration of the examined analytes with the patient data and clinical data collected by clinicians.

5.1.1. Characteristics of the study population

In total 85 participants were recruited in our study. Fasting serum was collected from all participants and tear samples were collected from volunteering patients with obesity or T2D. Clinical laboratory examinations were carried out on serum samples and the level of fasting glucose, HbA1C, triglyceride, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein A1 (ApoA1), apolipoprotein B100 (ApoB100), insulin, C peptide, C-reactive protein (CRP), fibrinogen and homocysteine (Hcys) was measured along with the glomerular filtration rate (GFR), albumin-to-creatinine ratio (ACR), and HOMA-IR. The circumferences of the abdomen (AC), waist (WC), and neck (NC) of the participants were measured and the body mass index (BMI) and the waist-to-hip ratio (WHR) were calculated using the proper formulas. All data from the clinical and laboratory analyses along with the demographic data (age, sex) are presented in **Table 4**. According to the American Diabetes Association (ADA), diabetes can be diagnosed either with a fasting blood glucose of ≥7.0 mmol/L or an HbA1c of ≥6.5%. In our study, the mean blood glucose level of the obese group was 5.3 mmol/L, while that of the T2D group ranged from 7.0 to 8.6 mmol/L. Furthermore, the average HbA1c was 5.5% in the case of the obese group and 6.6-7.5% in the case of the T2D group.

Table 4. Clinical characteristics of the subjects participated in serum and tear analysis.

Characteristics	Serum analysis (n=85)			Tear analysis (n=40)		
	Control (n=28)	Obese (n=31)	T2D (n=25)	Obese (n=19)	T2D (n=21)	
					non-DR (n=11)	DR (n=10)
Age (year)	55	53	54	55	53	55
Gender (M:F)	15:13	15:16	12:13	9:10	2:9	8:2
BMI (kg/m^2)	25	40	34	41	37	33
AC (cm)	N/A	133.5	123.4	131.7	125.1	124.8
NC (cm)	N/A	54.7	52.6	53.8	52.9	53.9
WC (cm)	N/A	141.4	129.8	141.7	140.3	121.7
WHR	N/A	0.9	1.0	0.9	1.0	1.0
Glucose (mmol/L)	N/A	5.3±0.5	8.2±2.7	5.3±0.5	8.6±2.7	7.0±1.1
Insulin (mU/L)	9.4±6.7	17.9±10.0	13.7±9.9	17.2±8.5	17.2±12.3	8.8±6.6
C-peptide	689±359	879±416	756±372	835±346	922±468	571±178
HbA1c (%)	5.5±0.5	5.5±0.3	7.2±1.1	5.5±0.3	7.5±1.2	6.6±1.0
HOMA-IR	N/A	4.3±2.8	5.1±4.2	4.1±2.3	6.5±4.8	2.6±1.9
ACR	0.7±1.3	1.7±2.1	4.7±4.8	2.7±2.6	2.2±1.8	7.3±5.9
GFR	79.7±8.5	88.8±3.4	86.0±9.0	88.6±3.7	87.8±4.8	83.7±13.3
ApoA1 (g/L)	1.7±0.3	1.6±0.3	1.6±0.3	1.6±0.3	1.5±0.2	1.7±0.3
ApoB100 (g/L)	1.1±0.2	1.2±0.3	1.1±0.3	1.2±0.3	1.2±0.3	1.1±0.2
Triglyceride (mmol/L)	1.2±0.7	1.7±0.7	2.6±3.5	1.7±0.8	4.3±4.9	1.3±0.5
Cholesterol (mmol/L)	5.5±0.9	5.4±1.2	5.1±1.1	5.7±1.4	5.7±1.2	4.7±0.8
HDL (mmol/L)	1.6±0.4	1.3±0.3	1.3±0.3	1.4±0.4	1.2±0.3	1.3±0.2
LDL (mmol/L)	3.6±0.8	3.8±1.0	3.3±0.9	4.0±1.2	3.6±1.0	3.2±0.7
CRP (mg/L)	3.3±4.4	6.5±5.6	6.1±5.8	5.9±5.0	7.4±8.1	5.3±2.3
Hcys (μml/L)	12.2±3.2	11.5±3.2	11.6±2.8	12.2±3.0	6.7±16.5	12.1±2.4
Fibrinogen (g/L)	3.8±0.7	4.2±0.6	4.5±1.0	4.1±0.7	4.4±1.0	4.6±0.9

Values are presented as mean±SD. N/A – not available, M – male, F – female

5.1.2. The concentration of the amino acids and biogenic amines in serum

A LC-MS analysis was carried out to determine the concentration of amino acids and biogenic amines in the sera. The analysis performed after pre-column derivatization using AccQ-Tag chemistry. The intensities of the peaks corresponding to each analyte were registered by the PDA detector at 260 nm as arbitrary units (AU) (**Figure 4**). The analytes were identified based on their retention time and the AUC was calculated for all peaks. Tryptamine and phenylethylamine could not be separated on the chromatograms and as the two compounds eluted in the same peak, could not be discriminated from each other. Mass spectrometry scans were used to verify the identity of the amino acids as well as to distinguish the co-eluting tryptamine and phenylethylamine.

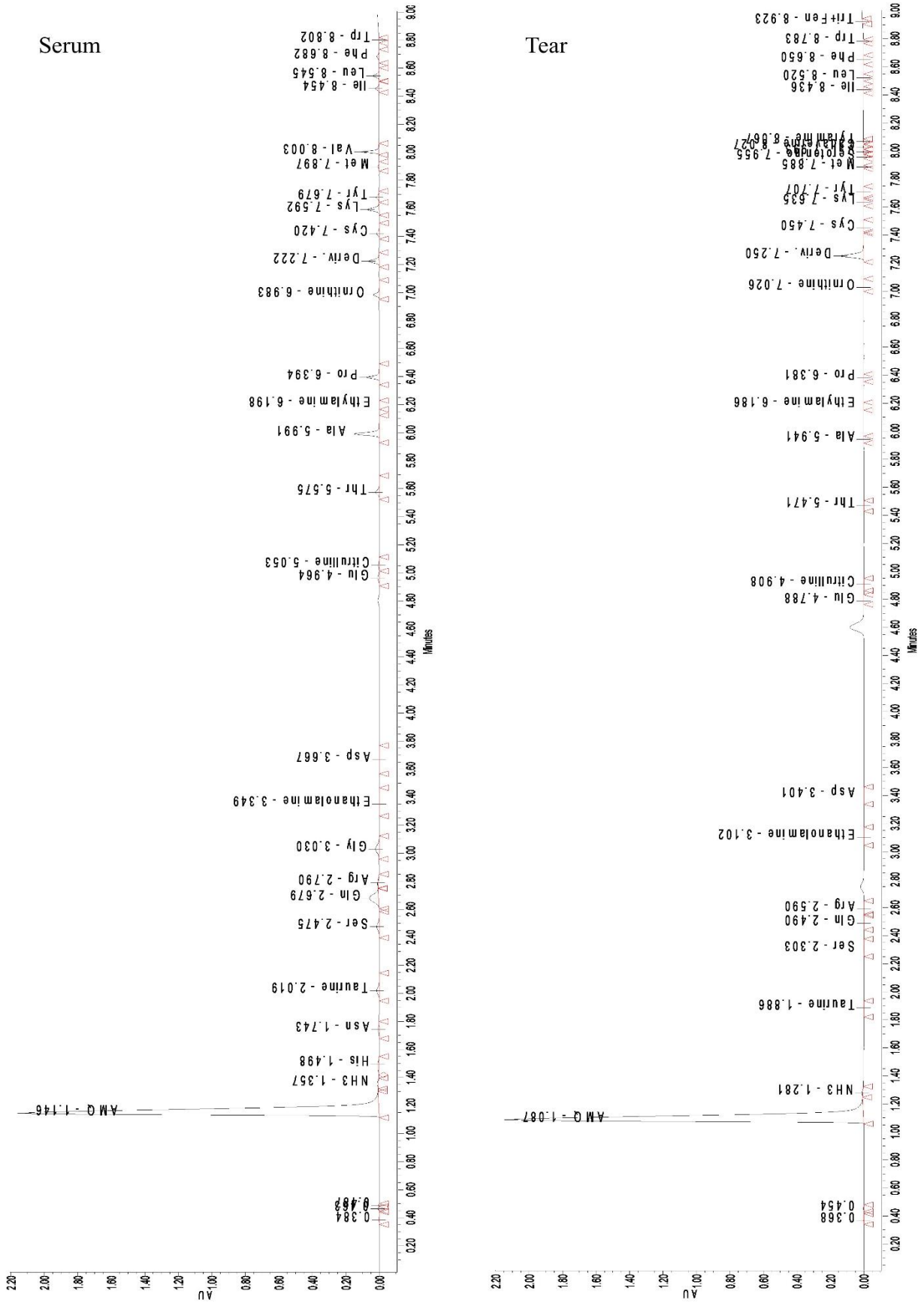


Figure 4. A representative PDA chromatogram of the studied amino acids and biogenic amines in serum and tear samples. The names of the analytes and the retention times are indicated for each peak.

5.1.2.1. Examination of the concentration of amino acids in serum

In our study, we detected and quantified all 23 amino acids analyzed in sera from donors belonging to control, obese and T2D groups (**Figure 5**).

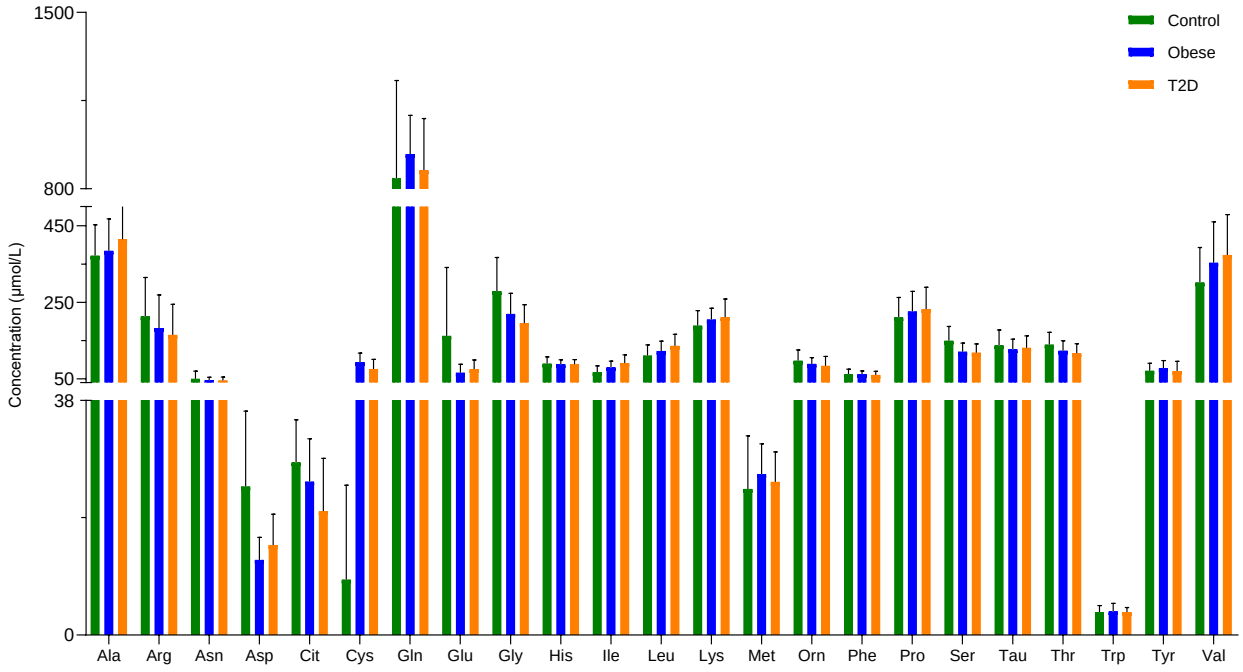


Figure 5. The concentration of amino acids in serum. The y-axis shows the concentrations of amino acids in micromol/L.

According to the bar chart in **Figure 5**, as expected, glutamine was found to be the amino acid with the highest concentration, while tryptophan was found to be the one with the lowest concentration in all groups. A statistical analysis was carried out to determine the statistically significant differences between our study groups. The results are shown in **Figure 6**.

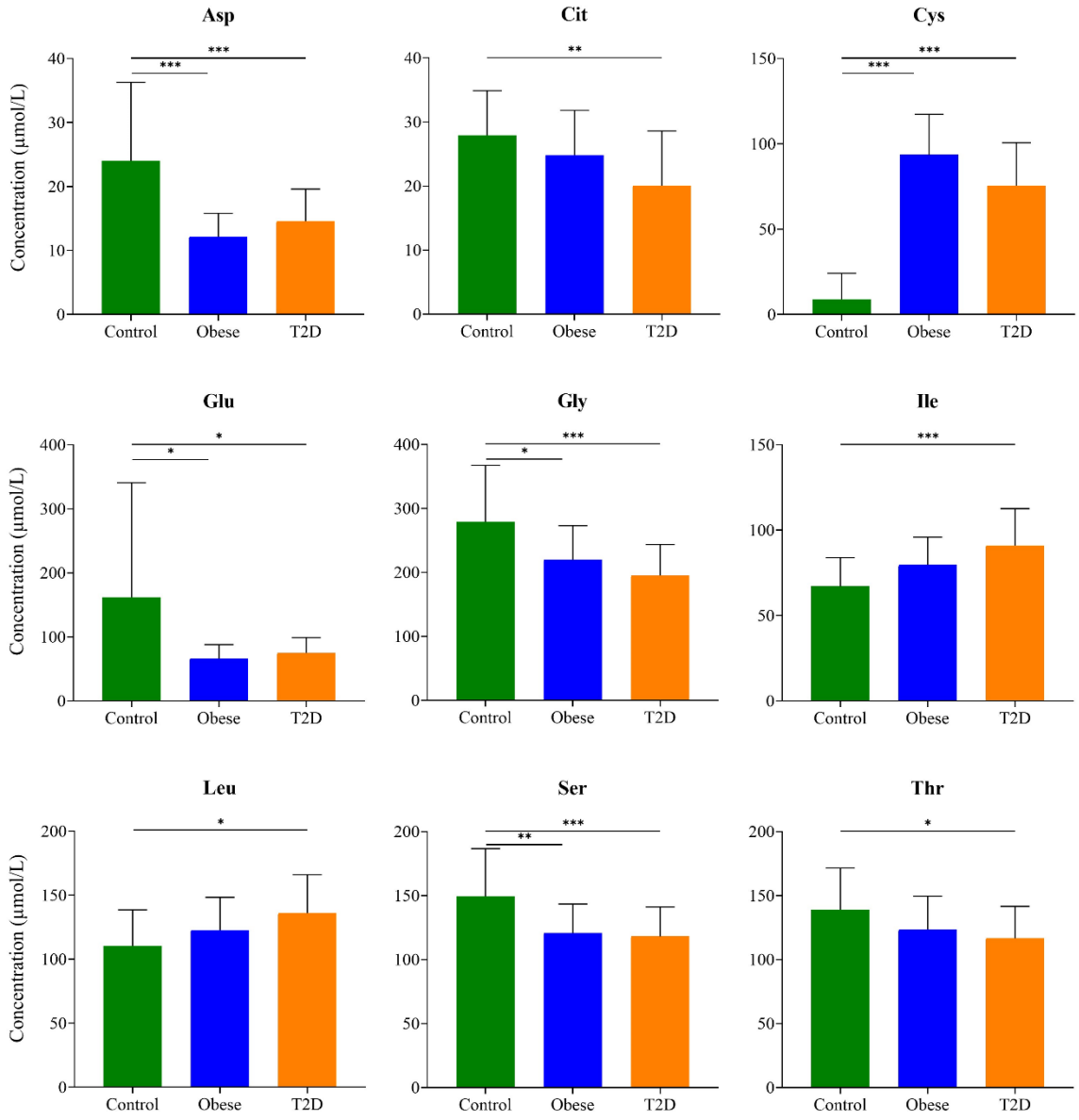


*Figure 6. The concentration of amino acids showing statistically significant changes between the study groups. The y-axis represents the concentrations of the individual amino acids, and the x-axis indicates the examined groups. *, p value ≤ 0.05; **, p value ≤ 0.01; ***, p value ≤ 0.001.*

The level of 9 amino acids showed statistically significant difference between the groups. A remarkable increase of cysteine and a decrease in the level of aspartate, glutamate, glycine, and serine was observed for the obese as compared to the control group. Similarly, in the context of T2D, we observed increased concentration of cysteine, isoleucine, and leucine and reduced concentration of aspartate, glutamate, glycine, serine, citrulline, and threonine as compared to the control group. When comparing the obese and the T2D groups no statistically significant differences were observed. These findings provide us valuable insights into the

metabolic perturbations associated with obesity and T2D, highlighting the potential roles of these amino acids in the pathogenesis of obesity and T2D.

5.1.2.2. Examination of the concentration of biogenic amines in serum

The levels of 10 biogenic amines were examined in the serum to reveal their relation with T2D and obesity. Ethylamine, putrescine, and serotonin were detected, but their level was lower than the limit of quantification. Therefore, we could only detect their presence in the serum.

Ethanolamine and methylamine were present in higher amounts in the serum. However, methylamine was quantified only in 6 samples out of 85, it was excluded from further statistical analysis and only ethanolamine was subjected to statistical analysis (**Figure 7**). The level of histamine, cadaverine, tyramine, and phenethylamine were under the detection limit of our method and these analytes were not detected in the serum.

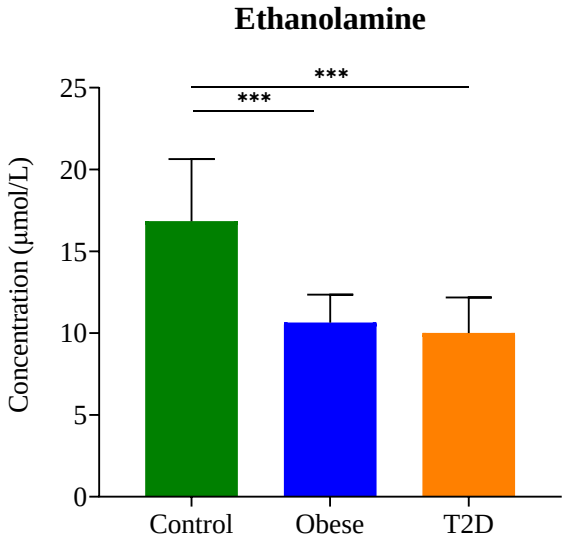


*Figure 7. The serum concentration of ethanolamine. The y-axis represents the concentrations of the biogenic amines in micromol/L. ***, p value ≤ 0.001.*

As presented in **Figure 7**, the concentration of ethanolamine was significantly lower in both T2D and obese groups as compared to the control group.

5.1.2.3. Correlation analysis

One of our main goals was to obtain more information about the involvement of the different amino acids and biogenic amines in the pathophysiology of obesity and T2D. To gain

deeper insights into the connections between clinical parameters and the levels of amino acids and biogenic amines in serum, we carried out Pearson correlation analysis (**Table 5**).

A range of significant correlations were identified between various metabolites and clinical parameters. Notably, a positive correlation was observed between BMI and serum cysteine levels, suggesting a potential link between body mass and cysteine metabolism. Conversely, aspartate, ethanolamine, glycine, and serine levels exhibited negative correlations with BMI, indicating an inverse relationship between these amino acids and the body weight. Moreover, triglyceride levels showed positive correlations with alanine, cysteine, isoleucine, and leucine, and a negative correlation with glycine levels.

Table 5. Correlation analysis of the clinical parameters with the examined serum amino acid and biogenic amine concentrations.

Clinical parameter	Serum analyte	Correlation coefficient (rho)	p-value	FDR-corrected q value
ACR	Eth	-0.41	0.00213	0.04408
ApoAI	Tyr	-0.34	0.00169	0.04027
BMI	Eth	-0.45	0.00006	0.00582
	Gly	-0.38	0.00077	0.02288
	Asp	-0.36	0.00166	0.04027
	Ser	-0.34	0.00251	0.04718
	Cys	0.64	0.00000	0.00000
C peptide	Gly	-0.43	0.00007	0.00593
	Ser	-0.37	0.00067	0.02093
CRP	Thr	-0.33	0.00225	0.04514
	Cys	0.36	0.00085	0.02392
Fibrinogen	His	-0.40	0.00029	0.01289
	Thr	-0.37	0.00092	0.02479
GFR	Eth	-0.42	0.00048	0.01831
	Gly	-0.38	0.00181	0.04134
HbA1C	Ile	0.35	0.00147	0.03804
Hcys	Cit	0.33	0.00237	0.04607
HDL	Leu	-0.50	0.00000	0.00036
	Ile	-0.49	0.00000	0.00043
	Val	-0.40	0.00016	0.00920
	Phe	-0.37	0.00052	0.01831
HOMA	Gly	-0.50	0.00012	0.00759
Insulin	Gly	-0.55	0.00000	0.00003
	Ser	-0.39	0.00028	0.01289
Trigliceride	Gly	-0.34	0.00186	0.04134
	Ala	0.34	0.00194	0.04158
	Cys	0.37	0.00061	0.01983

	Leu	0.37	0.00048	0.01831
	Ile	0.41	0.00012	0.00759
WHR	Val	0.45	0.00053	0.01831
	Leu	0.48	0.00024	0.01226
	Ile	0.51	0.00008	0.00605

The correlation coefficients, the p values and the FDR-corrected q values are shown for each statistically significant correlation.

Furthermore, the correlation analysis revealed a positive correlation between cysteine concentration and CRP levels, implicating cysteine in the context of inflammation. Conversely, threonine levels were negatively correlated with serum CRP, suggesting a potential anti-inflammatory role of threonine. Homocysteine exhibited a positive correlation with citrulline, while HbA1C a positive correlation with isoleucine, indicating potential metabolic relationships. WHR showed a positive correlation with the levels of branched-chain amino acids (BCAAs), i.e. with valine, leucine, and isoleucine. Negative correlations were identified between ApoA1 and tyrosine, HDL and phenylalanine, as well as HDL and BCAAs, implying complex associations between lipid, BCAA and aromatic amino acids metabolism.

Kidney function parameters such as ACR and GFR showed negative correlations with serum ethanolamine level, suggesting a potential link between renal function and ethanolamine metabolism. Additionally, serum ethanolamine levels exhibited a negative correlation with BMI, further highlighting its potential role in metabolic regulation.

Moreover, our correlation analysis revealed negative associations between serum C-peptide, glycine and serine, respectively. Likewise, negative correlations were found between glycine and HOMA-IR; fibrinogen and histidine, as well as fibrinogen and threonine, indicating an interplay between their metabolic pathways.

5.1.2.4. Network analysis

A network analysis was conducted in order to go beyond the classical statistical analysis and to identify the deeper connections. Two networks were generated; one for the obese group and another for the T2D group, taking into account the variations in the analytes that showed a statistically significant difference between the control group and obese group (**Figure 8**) as well as control and T2D groups (**Figure 9**).

Regarding the network characteristic to obesity, several distinct clusters have been observed such as the cluster of transporter proteins (SLC family members), one of VPS proteins and sorting nexins (SNX1, SNX2, SNX5, and SNX6) involved in retrograde transport from

Golgi to endoplasmic reticulum, and the cluster of proteins that are involved in iron-sulfur cluster assembly. Enzymes, related to energy generation: dihydrolipoamide branched chain transacylase E2 (DBT), malate dehydrogenase 1 (MDH1), and pyruvate dehydrogenase kinase 1 (PDK1), different forms of aldehyde dehydrogenase (ALDH), catalase (CAT), branched-chain ketoacid dehydrogenase (BCKDHB), ferredoxins (FDX1 and FDX2), sirtuin 4 (SIRT4), and sorting nexin 5 (SNX5), lipoyl transferase 1 (LIPT1) were present in the obese network.

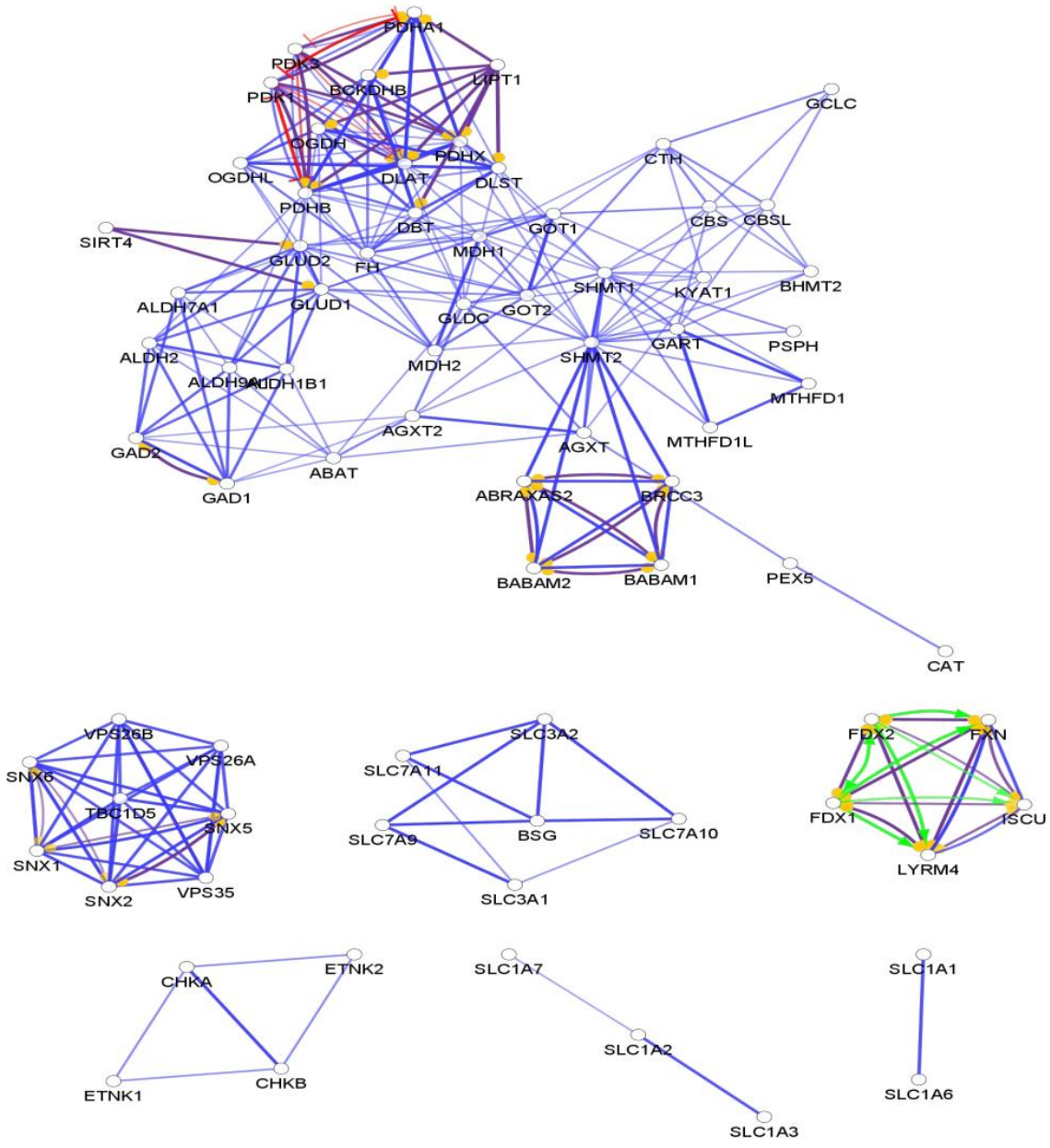


Figure 8. Gene interaction networks for obese group. Lines with various widths represent protein–protein interaction. Line color indicates the type of interaction: green, activation; red, inhibition; blue, binding; purple, catalysis. The proteins are labeled with their gene name.

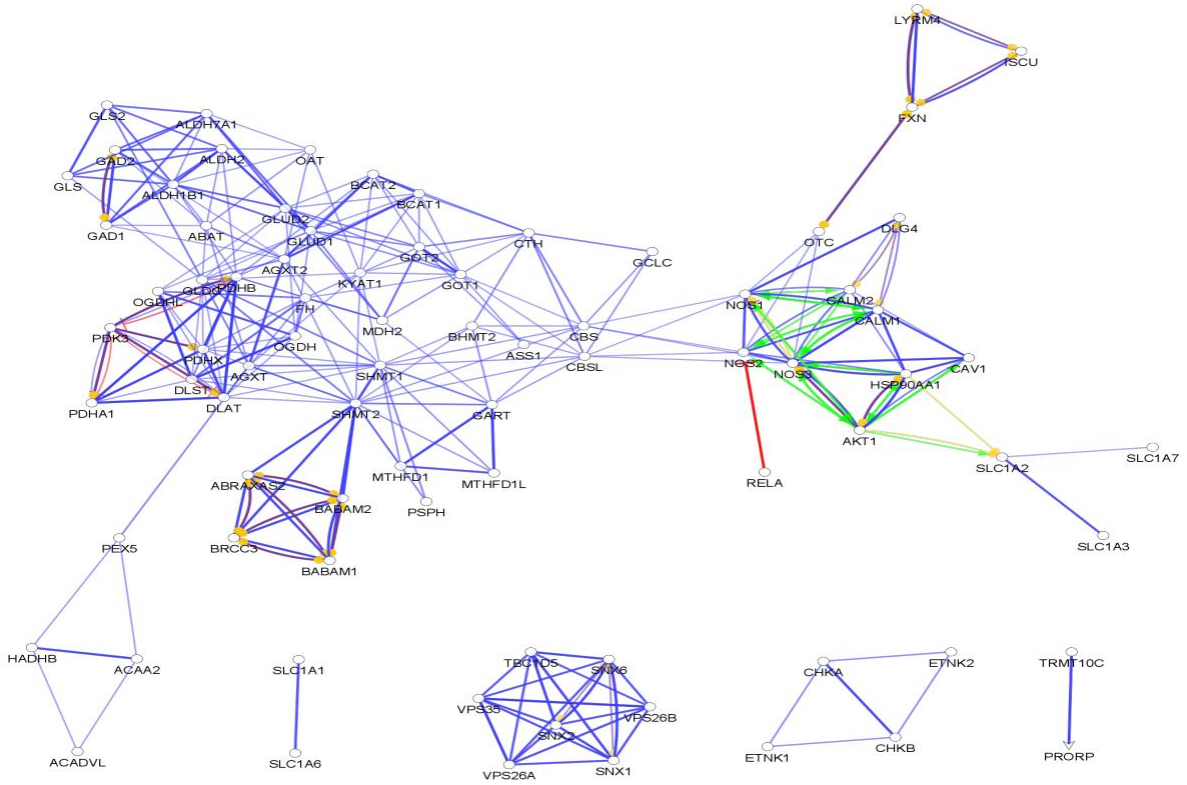


Figure 9. Gene interaction networks obtained for T2D. Lines with various widths represent protein–protein interaction. Line color indicates the type of interaction: green color refers to activation, red color to inhibition, blue color to binding, and purple color to catalysis. Proteins are labeled with their gene name.

Figure 9 shows the T2D network which has a substantial overlap with the previously illustrated obese network. In this network, we can see distinct clusters. The cluster of amino acids metabolism-related enzymes includes the argininosuccinate synthase 1 (ASS1), BCAA transaminases (BCAT1 and 2), glutaminases (GLS and GLS2), and ornithine aminotransferase (OAT).

Another characteristic cluster of the T2D network includes beta-oxidation enzymes, such as acyl-CoA dehydrogenase very long (ACADVL), acetyl-CoA acyltransferase 2 (ACAA2), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (HADHB). The NO synthesizing enzymes (NOS1, NOS2, and NOS3), calmodulins and caveolin 1 formed a highly interconnected cluster and was linked to SLC1A7 and SLC1A3 transporters. In this highly active cluster, calmodulin kinase 1 and 2 (CALM1 and CALM2) were mutually activating NOS1, 2, and 3; while NOS1 and heat shock protein 90 (HSP90AA1) were activating AKT, and in return, AKT activates SLC1A2 and caveolin 1 (CAV1). The cluster of iron-binding proteins includes frataxin (FXN), iron-sulfur cluster protein (ISCU), and

LYR motif-containing protein 4 (LYRM4) and was linked via ornithine transcarbamylase (OTC) to the NOS-containing cluster.

The enriched GO terms in the obese networks were retrieved using ClueGO v2.5.7 in order to have functional insights regarding the proteins (**Figure 10** and **Figure 11**).

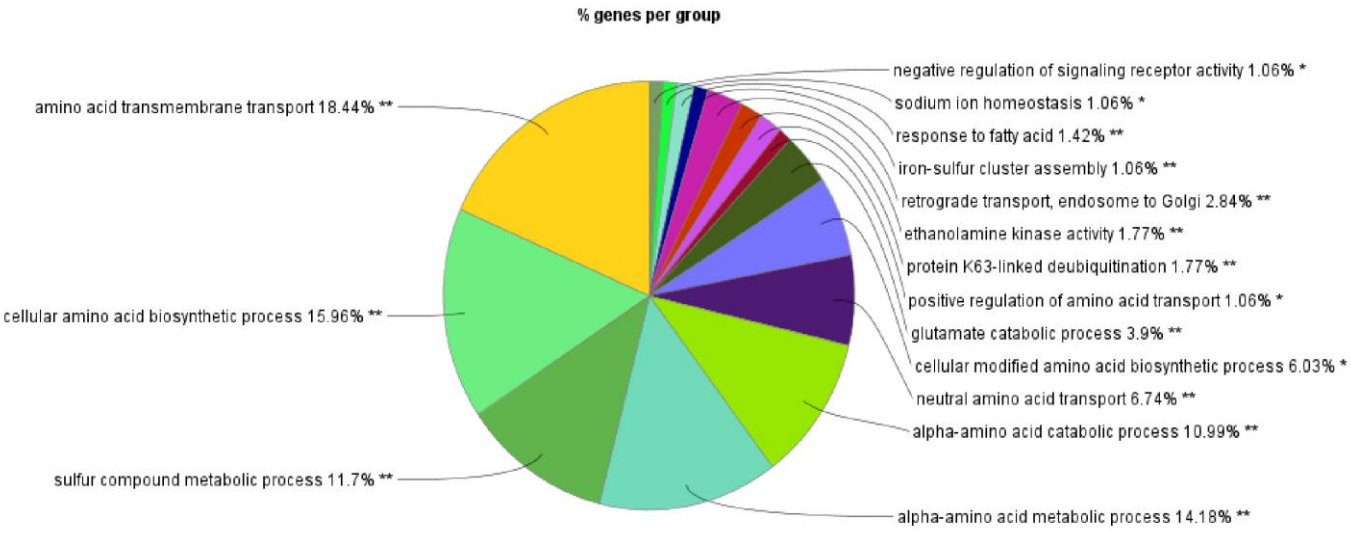


*Figure 10. Significantly enriched GO terms in obese network. * $p \leq 0.05$; ** $p \leq 0.01$*

As expected, many of the enriched GO terms were related to amino acid transport and metabolism. According to the GO terms, the response to fatty acid, iron-sulfur cluster assembly, and sulfur compound metabolic process was found to be characteristic functions for the obese network.

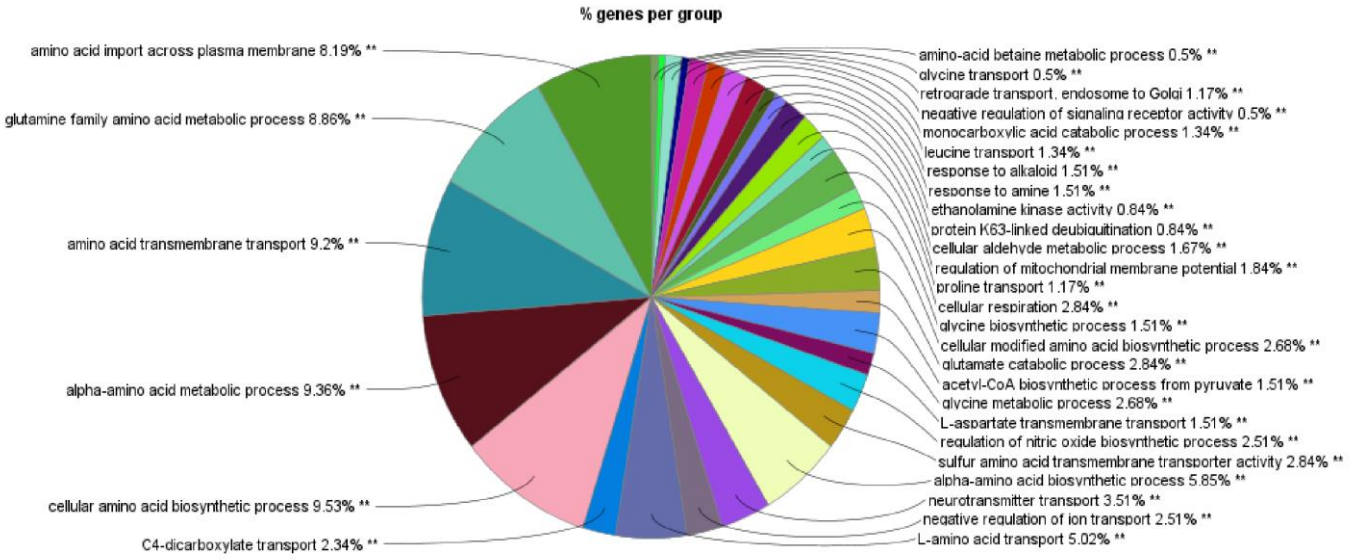


*Figure 11. Significantly enriched GO terms in T2D network. * $p \leq 0.05$; ** $p \leq 0.01$*

The characteristic functions of T2D were mainly associated with the metabolism of glycine and glutamine, response to amine and alkaloid, degradation of monocarboxylic acid, aldehyde metabolism, cellular respiration, C4-dicarboxylate transport, generation of acetyl-CoA from pyruvate and regulation of nitric oxide synthesis.

The cytoHubba plug-in of the CytoScape software was applied to visualize the top 20 hub proteins, in order to acquire further insights into the critical proteins (**Figure 12**).

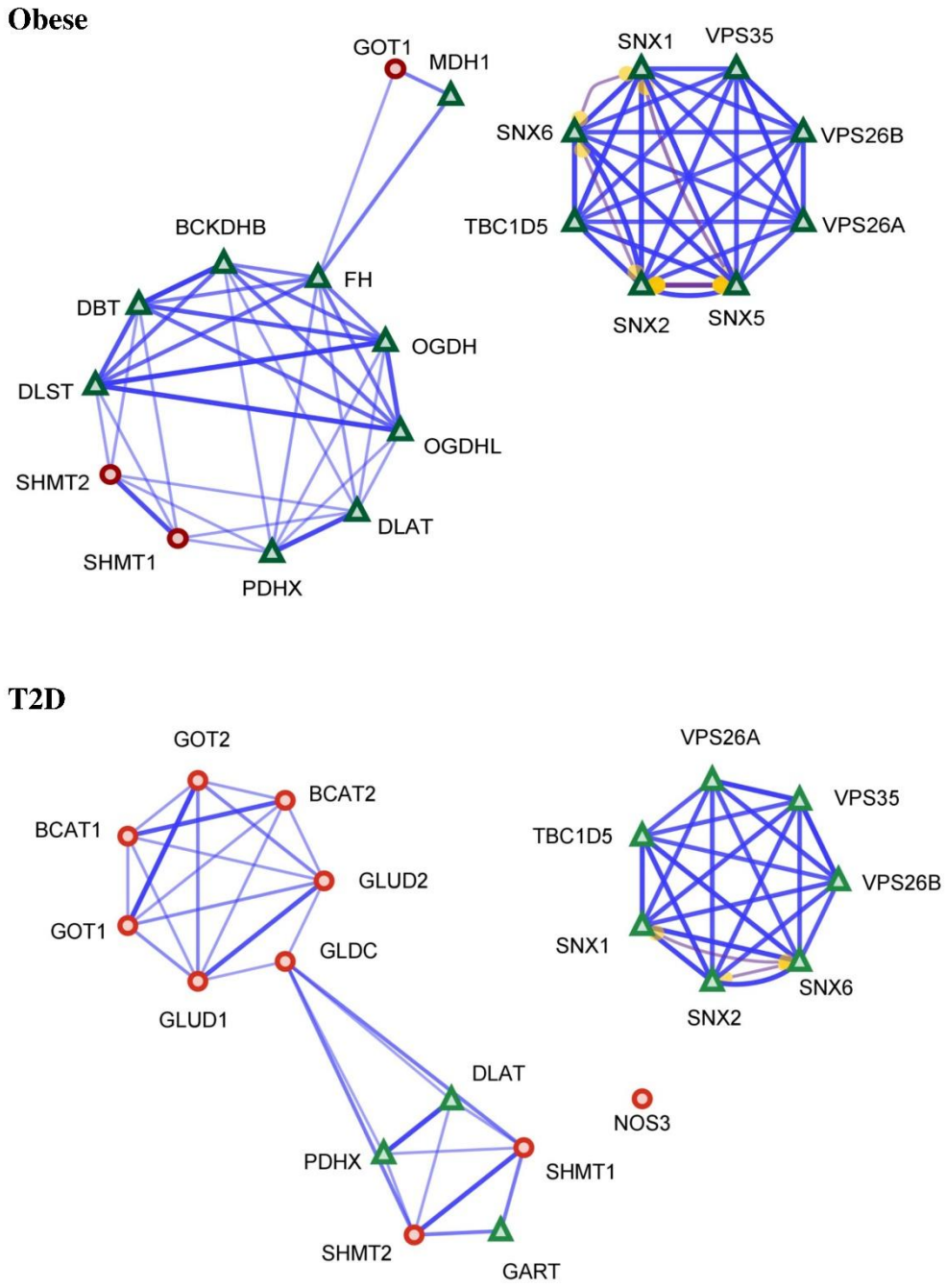


Figure 12. Top 20 hub proteins and their interactions in obese and T2D networks. The red circles represent the enzymes and/or transporters belonging to metabolic pathways of the

analytes with statistically significant difference between the groups, while the green triangles represent their first shell interactors. Lines with various widths represent protein–protein interaction. Line color indicates the type of interaction: blue color refers to binding, and purple color to catalysis. The genes or proteins are labeled according to their gene names.

These hub proteins align with the results obtained from the analysis of the Gene-interaction Network. The proteins that exhibited strong connections in the obese network were specifically associated with energy production. These proteins include dihydrolipoamide S-succinyltransferase (DLST), fumarate hydratase (FH), oxoglutarate dehydrogenase (OGDH), OGDHL, MDH1, DBT, and BCKDHB. The T2D network prominently featured enzymes involved in amino acid metabolism, including BCAT1, BCAT2, glycine decarboxylase (GLDC), glutamate dehydrogenase 1 and 2 (GLUD1 and GLUD2), glutamic-oxaloacetic transaminase 2 (GOT2), nucleotide metabolism-related enzyme (GART), and NO synthase 3 (NOS3) in central positions of the network. The highly interconnected cluster of proteins involved in retrograde transport had a central role in both networks, the only difference being SNX5, which was missing from the T2D network.

5.1.3. Examination of tear metabolome

Tear was collected from patients who agreed to donate tear samples. In this way, our setup allowed only for the comparison of tear metabolites between the obese and T2D groups. The patients with T2D were divided into two distinct groups, DR and non-DR based on ophthalmological examination by specialists. In this way, we had three groups: obese, T2D with DR (DR) and T2D without DR (non-DR).

Similarly to the examination of amino acids and biogenic amines on serum, tear samples were also studied. All the studied amino acids were identified and all but methionine were quantified in all tear samples (**Figure 13**). Methionine was quantified in the obese group and detected but not quantified in the tears of T2D patients. Contrary to the serum, the concentration of serine and glycine were the highest in tear. Subsequently, no statistically significant differences were found among the studied groups.

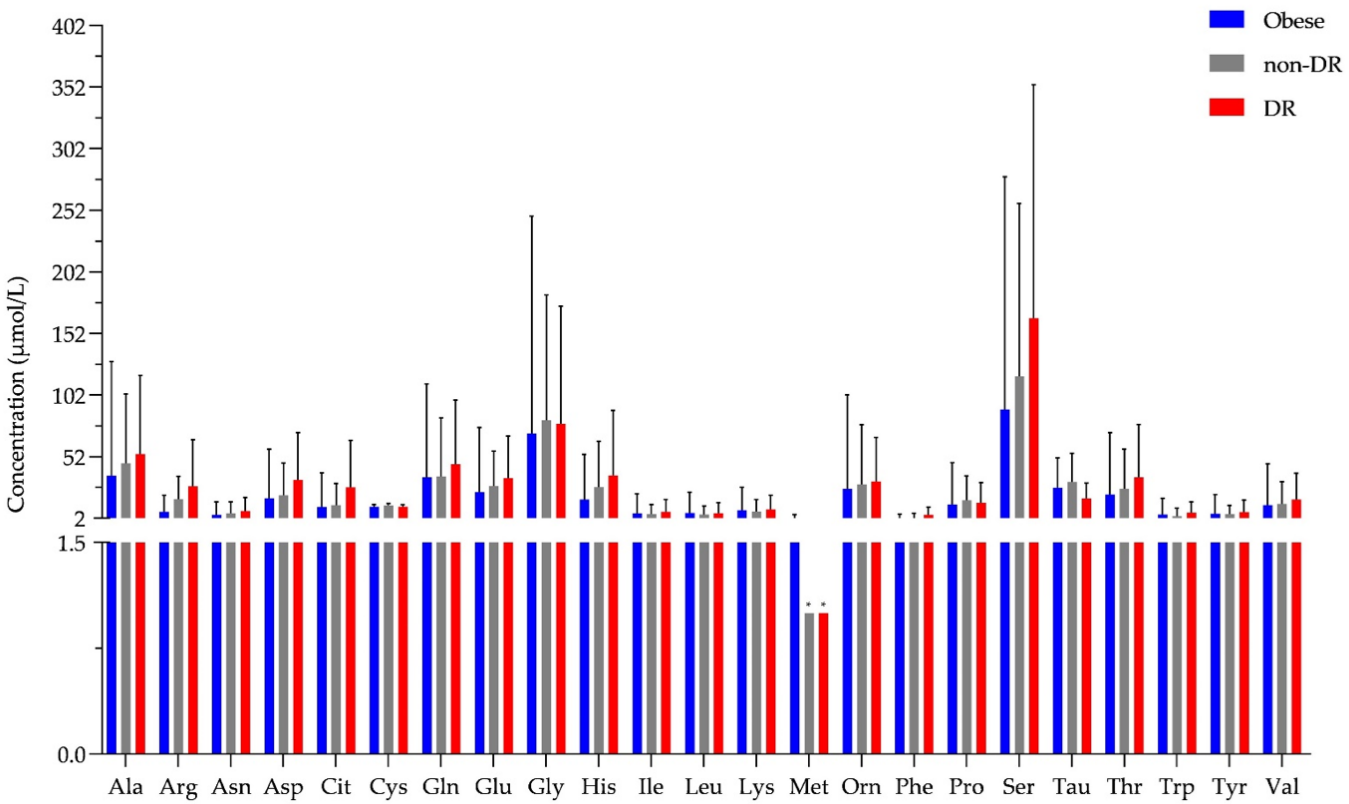


*Figure 13. The concentrations of amino acids in tear. The y-axis shows the concentrations of the amino acids in micromol/L. *The Met in the DR and non-DR groups was detected but was not quantified, in these cases the value corresponding to the limit of its quantification was plotted.*

9 of the studied 10 biogenic amines were successfully identified in tears. We could quantify only ethanolamine, but no statistically significant difference was observed between groups, unlike in the serum analysis (**Figure 7**).

The correlation analyses - which were performed previously on serum, as well - were conducted on tear metabolites, but none of the analytes exhibited statistically significant correlations with the clinical data.

5.2. Comparative analysis of grape-derived products

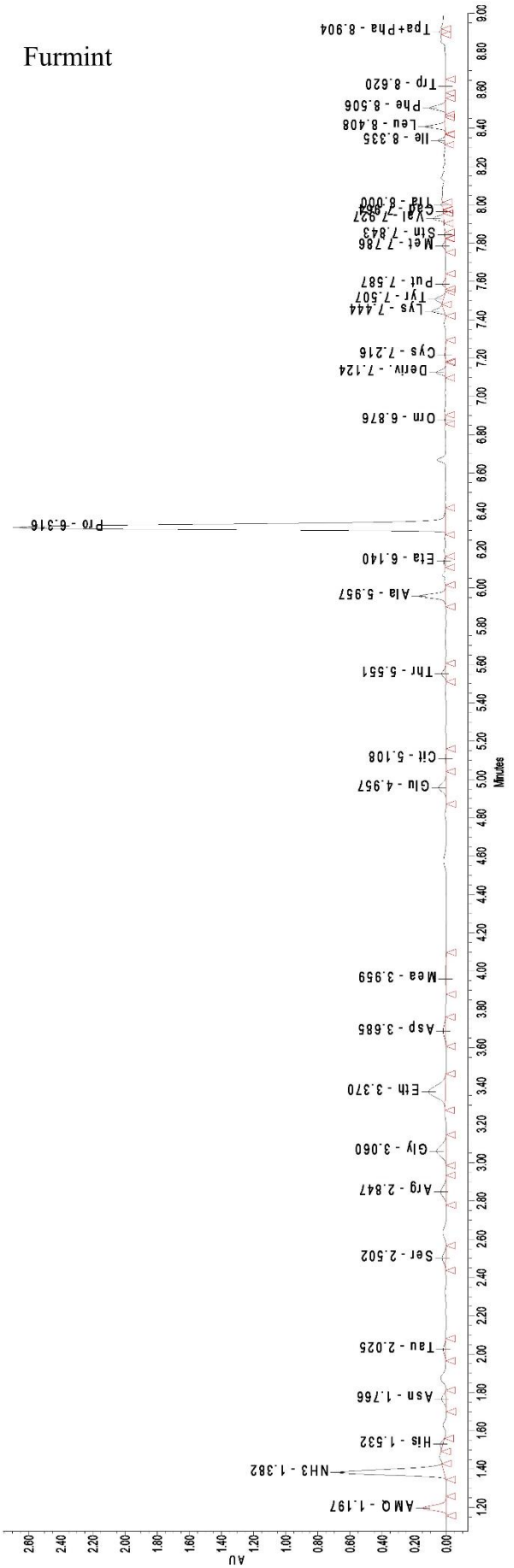
In this study, we examined the concentrations of 23 amino acids and of 10 biogenic amines in Furmint, Aszú, Essence, and wine vinegar samples originated from Tokaj region, a Hungarian wine region, part of UNESCO World Heritage. By utilizing a sensitive and high-throughput UHPLC-MS system 8 Furmint, 8 Aszú, 2 Essence, and 4 wine vinegar samples were analyzed.

5.2.1. Analysis of the concentration of amino acids and biogenic amines in examined grape-derived products

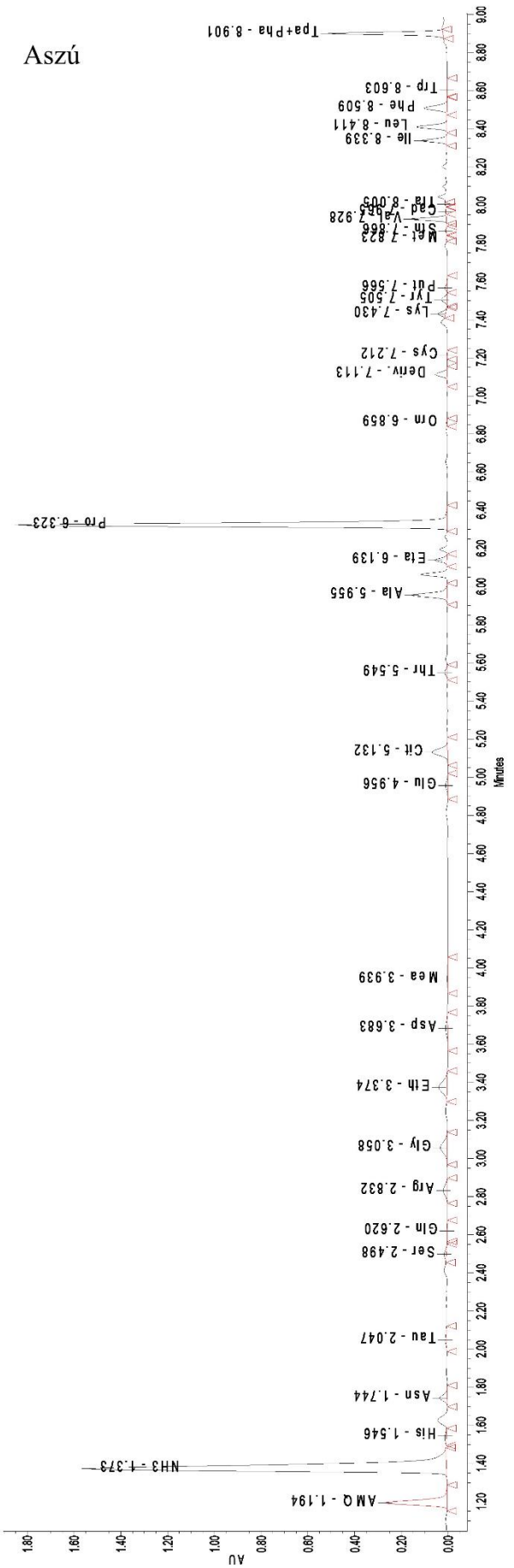
An LC-MS analysis was carried out in the case of the four types of grape-derived products (**Figure 14**), similar to the analysis of human serum and tear samples.

All the examined 23 amino acids were detected and quantified both in the Aszú and the Furmint samples, while glutamine, citrulline, and tryptophan were not detected in wine vinegar samples (**Figure 15**).

Furmint



Aszú



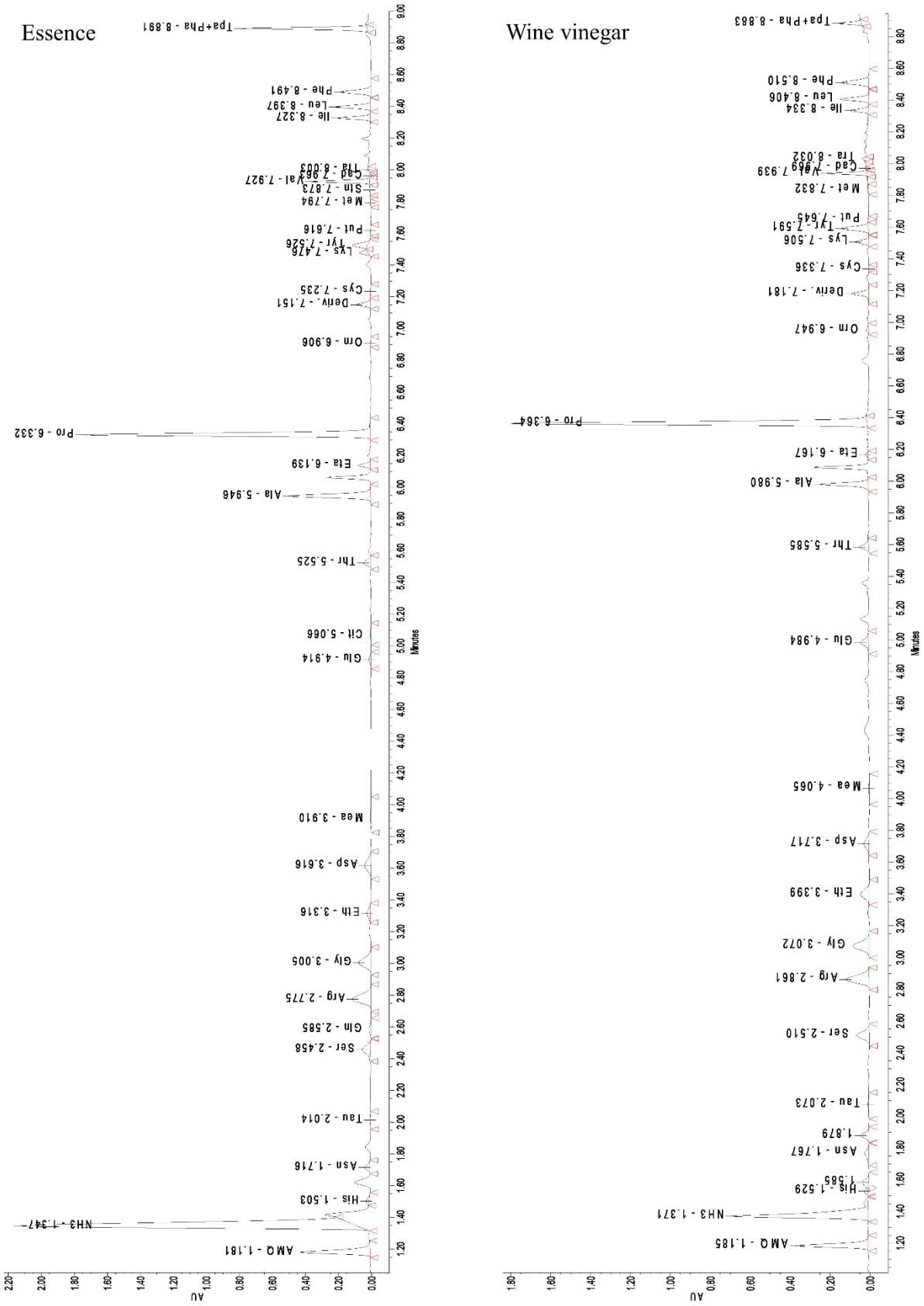


Figure 14. Selected chromatograms of the studied amino acids and biogenic amines in Furmint, Aszú, Essence, and wine vinegar. The x axis shows the retention time in minutes while the y axis shows the intensity (AU). Amino acids are labeled with their 3-letter code, AMQ – 6-aminoquinolone, Deriv. – Derivatization peak.

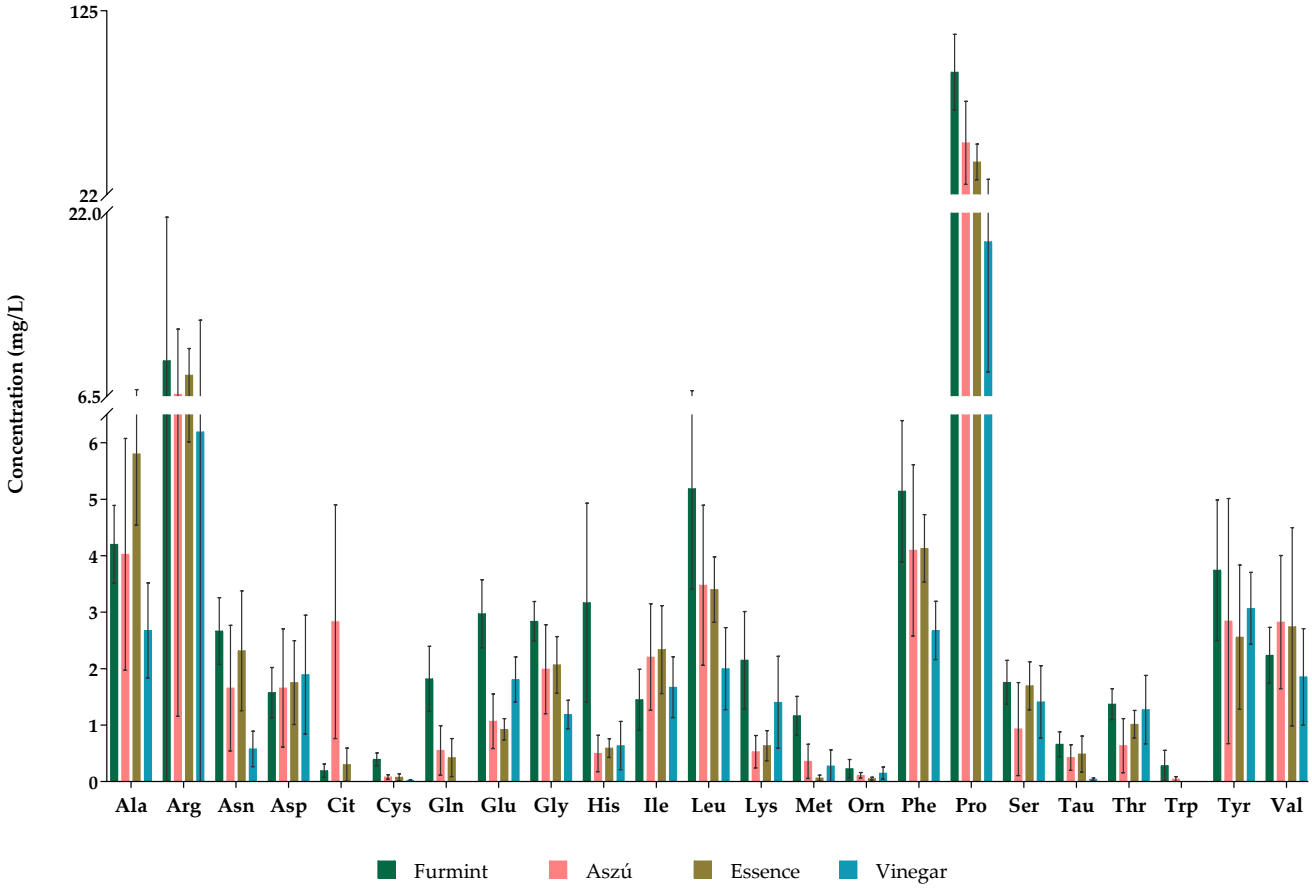


Figure 15. The concentrations of amino acids in all studied grape-derived beverages. The y-axis shows the mean concentrations of the detected amino acids in mg/L and the standard deviations (SD).

Proline was found to have the highest concentration in all sample types. The concentration of proline in Aszú was between 19.6 - 103.7 mg/L, in Furmint between 53.9 - 119.9 mg/L, in Essence between 31.5 - 48.9 mg/L, whereas, it ranged between 10 - 37.1 mg/L in wine vinegar (**Figure 15**). In addition to the proline, the arginine, alanine, and phenylalanine were present in relatively high concentrations in all studied wine samples, the arginine was one of the most abundant amino acids in vinegar. Conversely, cysteine and ornithine were found to be present at the lowest concentrations across all examined samples. In Essence, all amino acids except tryptophan were detected. Ornithine, cysteine, and methionine were detected, but not quantified, as their concentration was lower than the limit of quantification. Overall, the levels of amino acids were higher in Furmint than in the other sample types.

All examined biogenic amines except histamine were detected in the analyzed samples (**Figure 16**).

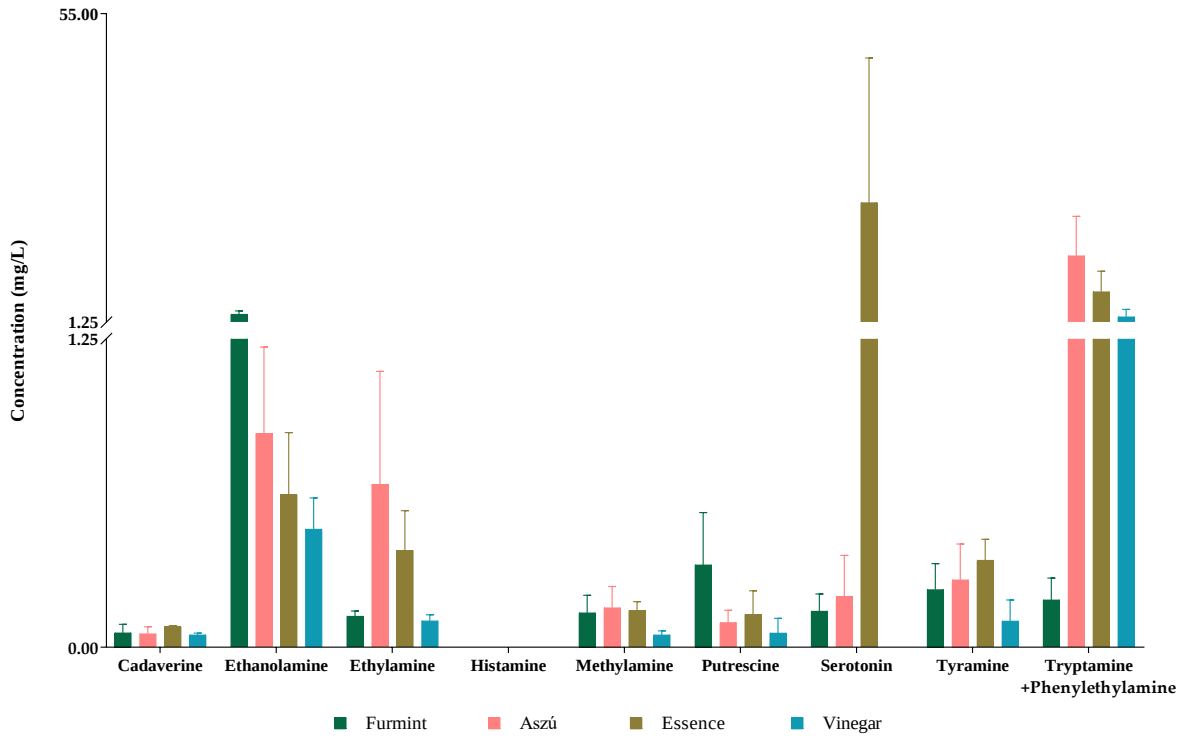


Figure 16. The concentration of biogenic amines in all studied grape-derived beverages. The y-axis shows the concentrations of biogenic amines in mg/L.

5.2.1.1. Comparative analysis of wine and wine vinegar samples regarding their amino acid content

The comparative analysis between different samples showed significant differences in the concentration of 18 amino acids (**Figure 17**).

The concentrations of cysteine, glutamine, glutamate, glycine, histidine, lysine, methionine, proline, serine, taurine, threonine, and tryptophan were significantly higher and of citrulline was significantly lower in Furmint as compared to Aszú. The concentrations of cysteine, glutamine, glutamate, lysine, methionine, ornithine, tryptophan, and proline were significantly higher in Furmint as compared to Essence. Both Aszú and Essence are made from botrytized grapes. As we expected, we could observe similar amino acid profiles for these two sample types. The concentrations of alanine, asparagine, citrulline, cysteine, glutamine, glutamate, glycine, histidine, leucine, methionine, phenylalanine, proline, tryptophan, and taurine were higher in Furmint as compared to wine vinegar samples. The concentrations of cysteine, glycine, and proline were significantly lower, and of glutamate were higher in wine vinegars than in the Aszú, while no statistically significant difference was detected between Essence and vinegars.

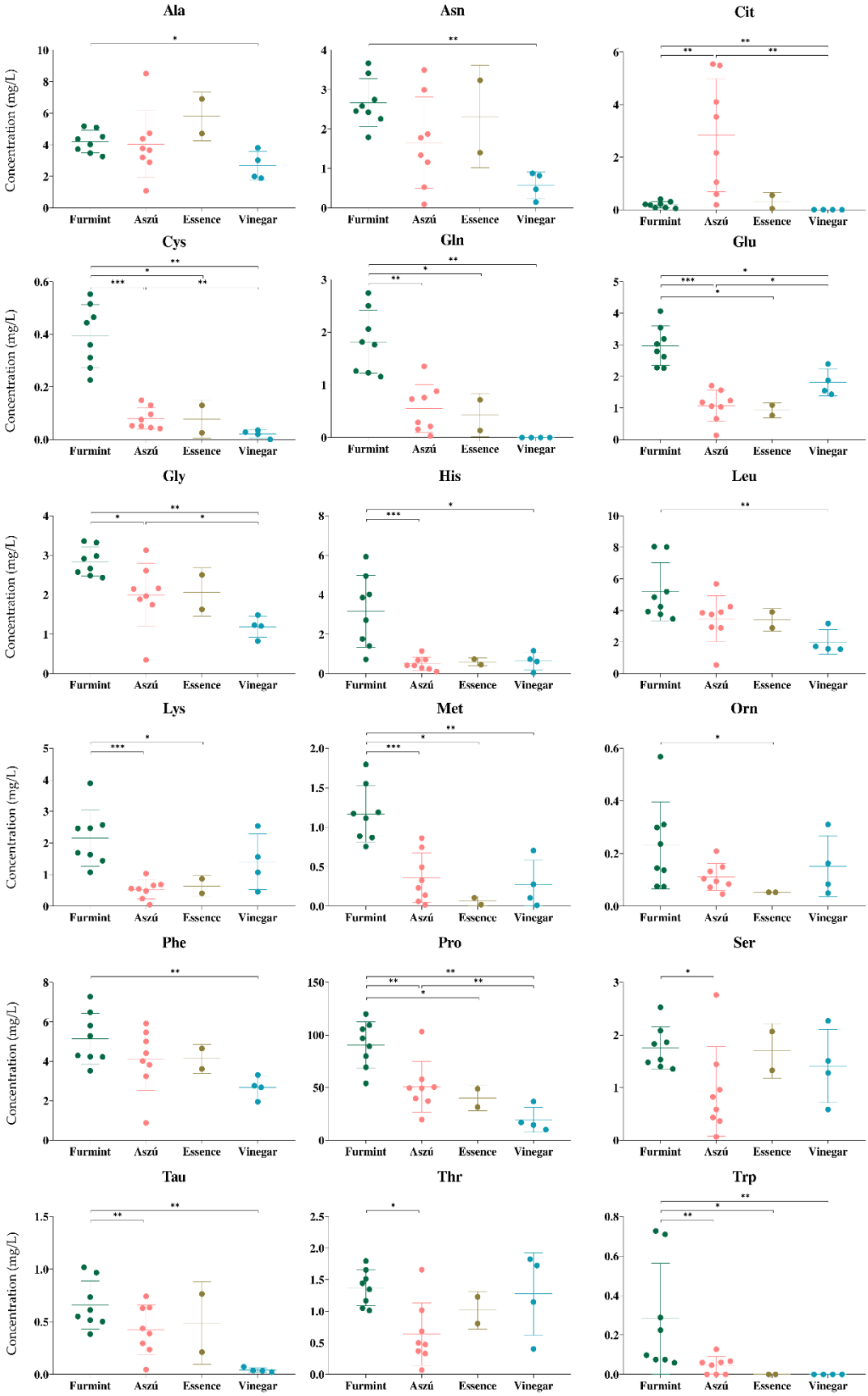


*Figure 17. The concentration of amino acids showing statistically significant differences between the groups. The y-axis shows the concentrations of the amino acids in mg/L and the SD in the four types of beverages examined. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.*

5.2.1.2. Comparative analysis of wine and wine vinegar samples regarding their biogenic amine content

In order to get information on beverage quality as well as the difference between the products in terms of their biogenic amines content, we examined the amount of 10 biogenic amines (**Figure 18**).

Among the different samples, we observed statistically significant changes in the quantities of five biogenic amines. Tryptamine and phenylethylamine we omitted from further examinations because the individual amounts could not be determined.

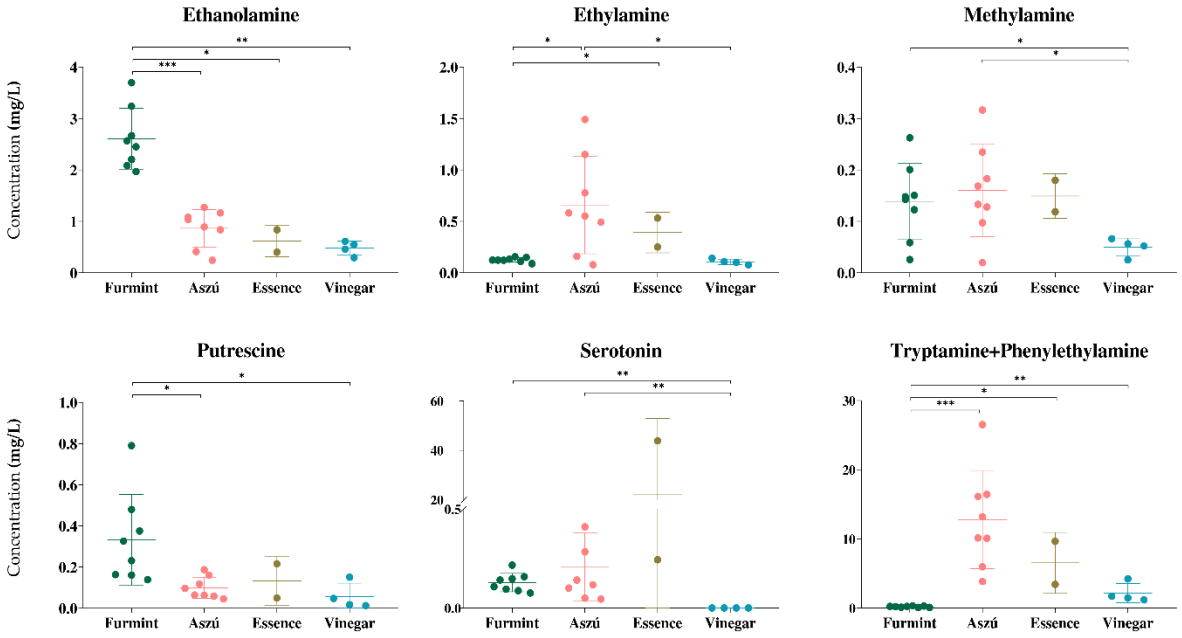


*Figure 18. The concentration of the biogenic amines showing statistically significant differences between the groups. The y-axis shows the concentration in mg/L of individual biogenic amines and SD in four types of beverages. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.*

Statistically significantly higher concentrations of ethanolamine and putrescine and lower concentration of ethylamine were found in Furmint as compared to Aszú. Furmint and Essence showed statistically significant differences only in the case of serotonin, ethanolamine and ethylamine. The concentration of ethanolamine was higher, while those of the serotonin and ethylamine were lower in Furmint than in Essence. The comparison of wine vinegar and wine samples revealed higher concentrations of ethanolamine, methylamine, putrescine, and serotonin in Furmint, and of ethylamine, methylamine, and serotonin in Aszú. Wine vinegar

had the lowest concentrations of cadaverine, methylamine, putrescine, and tyramine among all the studied samples.

5.2.2. Discrimination of sample types based on their amino acid and biogenic amine content

In order to examine if the sample groups can be discriminated based on their amino acid and biogenic amine content, principal component analysis (PCA) was carried out (**Figure 19**).

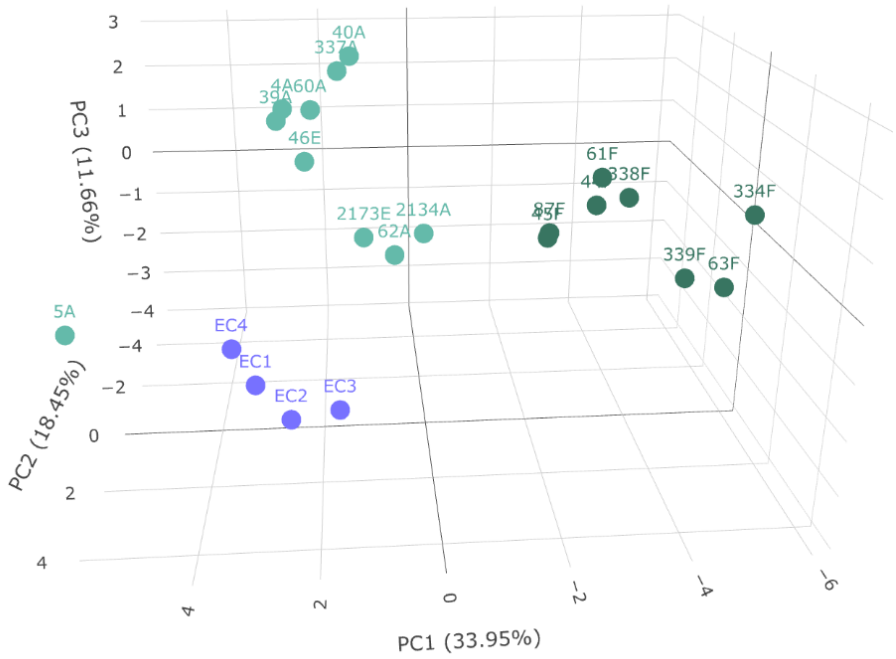


Figure 19. PCA of grape-derived beverages. Variance explained by three principal components (PC1-3) is indicated in axes labels. Dots in dark green refer to Furmint, the ones in light green to Aszú and Essence, while purple shows wine vinegar samples.

According to the PCA results, three groups could be distinguished from each other. The group of Furmint and of wine vinegar samples could be observed as distinct groups. The Aszú and Essence samples formed another group, with two subgroups. The Aszú and Essence samples did not separate from each other. Both Aszú 2134A and Essence 2173E samples belonging to the same subgroup, were manufactured in the year 1940. Regarding the Furmint group, one subgroup consisted of wines manufactured in 2013, except for 87F which was produced in 2016. The second subgroup included wines from 2017, and one from 2008. Surprisingly, sample 5A was an outlier, despite the fact that considering the individual analytes there were no outliers. This was an Aszú wine from the year 2000, labeled as 5-basket (**Table**

1). The results of the PCA analysis align with the findings shown in **Figure 17** and **Figure 18**, respectively. Unfortunately, it was not possible to distinguish the wineries, and the data on the terroir was not consistently available for all wines.

Clustering the results obtained from the analysis of amino acids and biogenic amine content of grape-derived beverages grouped the samples into two main clusters (**Figure 20**).

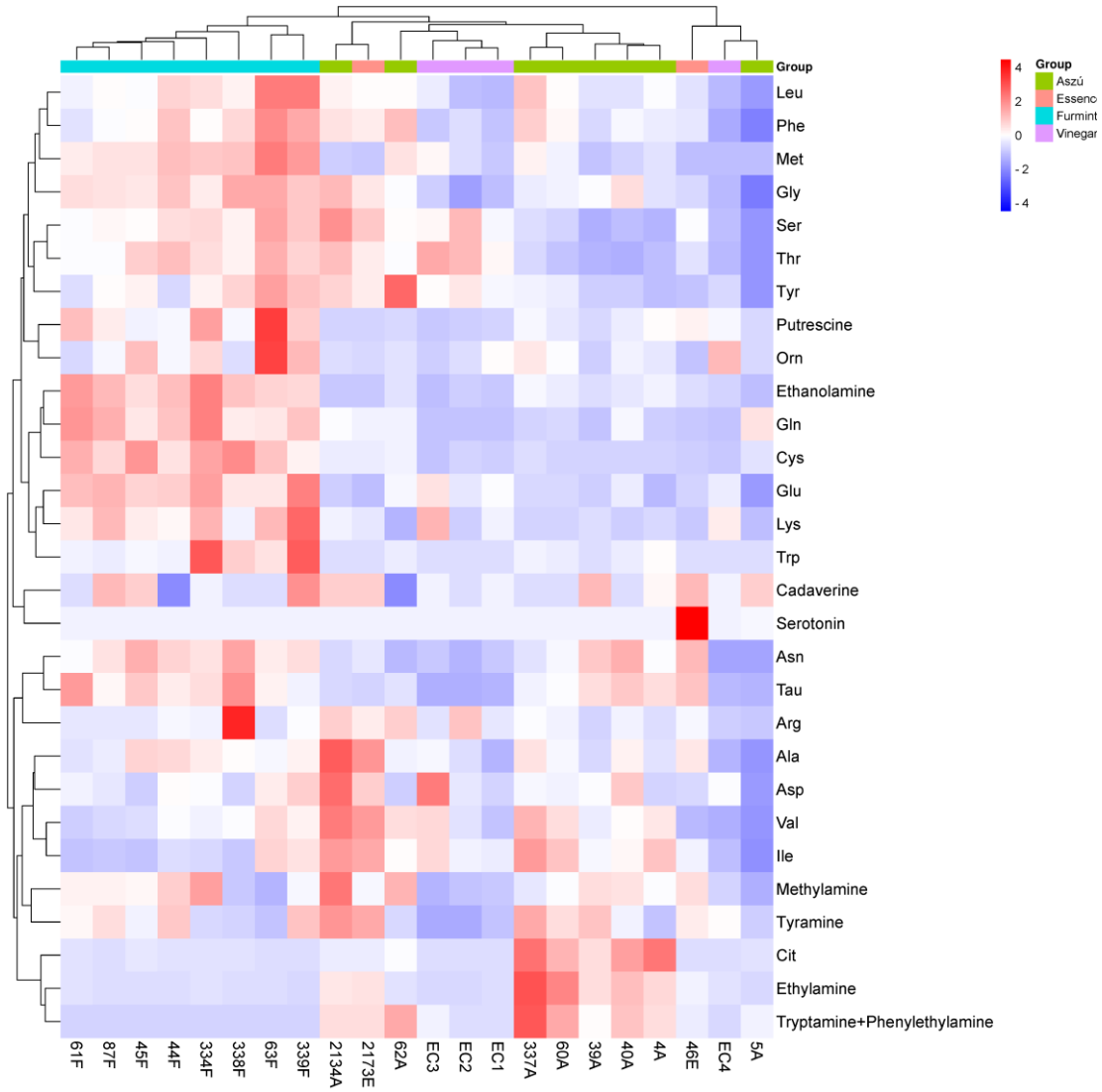


Figure 20. Heatmap analysis combined with hierarchical clustering of amino acids and biogenic amines in the examined grape-derived samples.

All Furmint samples were grouped into one cluster, whereas the other three samples were classified into different subgroups. The amino acid proline and biogenic amine histamine were excluded from the graph due to their very different concentrations (the level of proline was very high while the histamine was absent from the studied samples) which would distort the heatmap.

It was possible to differentiate sample types, however, a larger number of samples would be analyzed to differentiate based on the year, winery, and terroir more accurately.

5.2.3. Examination of the effect of botrytized grapes on wine metabolite content

As far as the process of Aszú production both Furmint and botrytized berries are involved, we did a comparative analysis of Furmint-Aszú pairs originating from the same winery and year (**Table 1**). In order to reveal the effect of botrytis on the analytes of Aszú and Furmint, the paired-t test was performed (**Figure 21**).

We observed statistically significant differences in the concentration of 11 amino acids and 3 biogenic amines between Furmint and Aszú sample pairs. The trend of changes in the concentrations of these 14 analytes was consistent, pointing in the same direction, indicating the impact of botrytized grapes on these metabolites. Furmint showed predominantly higher concentrations of cysteine, glutamine, glutamate, glycine, histidine, lysine, methionine, serine, threonine, tryptophan, ethanolamine, and putrescine than Aszú, while the levels of only citrulline and ethylamine were lower in Furmint. These findings suggest that amino acids present in lower concentrations might be used or transformed, while citrulline and ethylamine could be synthetized during or as a result of the infection of berries with *Botrytis sp*. The other amino acids and biogenic amines showed less consistent and no statistically significant differences, suggesting that other factors might contribute to the observed alterations in their concentration.

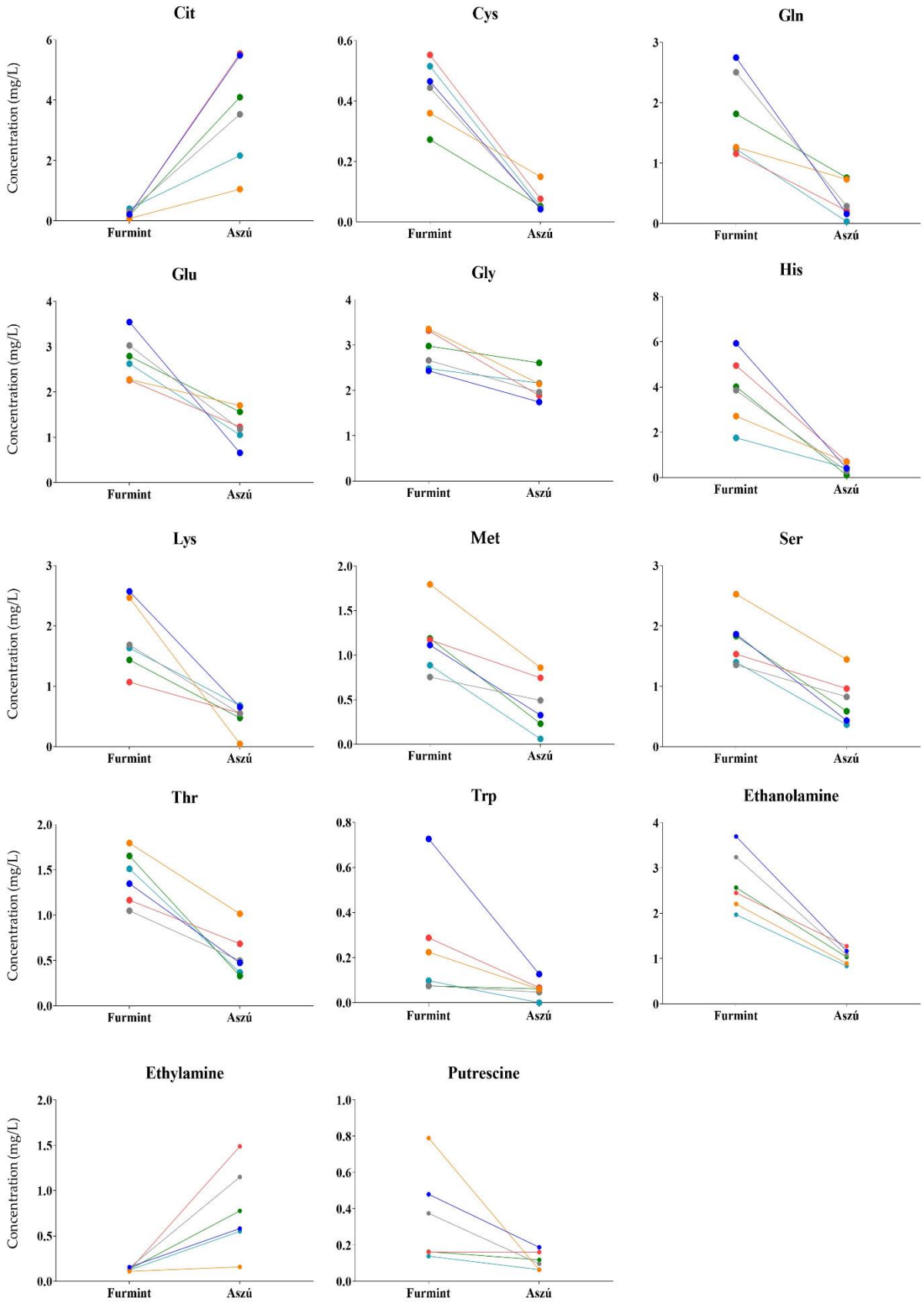


Figure 21. Comparative analysis of Furmint-Aszú pairs. The y-axis represent the concentrations of those amino acids and biogenic amines that showed statistically significant difference between Furmint and Aszú.

6. DISCUSSION

6.1. Examination of amino acids and biogenic amines in biological fluids in regard to obesity and type 2 diabetes

T2D and obesity can be considered as civilization diseases with such pathological manifestation that affect the life quality of millions of people worldwide [145,146]. Metabolomics includes commonly applied analytical approaches for profiling and studying alterations of the metabolism in relation to numerous disorders [20,88,147–149]. Analysis of metabolites that appear in the blood serum is a well-established technique for diagnosis and prediction of the development of T2D and obesity [150,151]. Examining the lipid profile and measuring the levels of certain proteins and small molecules is a standardized diagnostic procedure which can be used for monitoring the health status and identifying the different stages of diabetes or its asymptomatic progression [69,74,99].

In this study, chromatographic and mass spectrometric analysis of amino acids and biogenic amines in serum and tear samples collected from healthy individuals, patients with obesity, and patients with T2D was carried out. The data obtained were in line with literature data. According to our results, patients with T2D had higher concentrations of cysteine, isoleucine, and leucine as compared to healthy individuals, while the concentrations of aspartate, citrulline, glutamate, glycine, serine, and threonine, as well as ethanolamine, were lower. In addition, we found significantly lower concentrations of aspartate, glutamate, glycine, and serine in the case of patients with obesity as compared to healthy individuals. We found significantly decreased levels of serum aspartate in the groups obese and T2D subjects as compared to the control group. This result was similar to the outcomes of a previous study carried out by Chen *et al*. [149]. However, Zhou *et al.* found a significantly higher concentration of aspartate in the case of the obese group as compared to a lean group, even though their result were consistent with ours regarding the comparison of the diabetes group to the non-diabetes one [152].

In our study, cysteine had significantly higher concentrations in the sera of patients with obesity and T2D as compared to healthy individuals. This result corroborates the results of other researchers, in the case of the obesity versus lean group, while they didn’t find any difference between the T2D and nondiabetes groups [152]. In addition, other researchers didn’t measure the level of cysteine, although, they revealed many other amino acids being associated with these conditions [73,153]. However, Jain *et al*. found a significantly lower concentration of

serum cysteine in the case of T2D versus age-matched healthy controls [154]. These inconsistencies may arise from the analytical procedures applied for cysteine measurement. The alteration of cysteine level may be related to insulin resistance and if validated, cysteine can be an early biomarker of insulin resistance [155].

According to some literature data, serum glutamate levels were significantly increased in both obesity and T2D as compared to controls [74,152,156–159]. However, Drabkova *et al*. didn't find any significant difference in the level of glutamate between the control group and patients with T2D [153]. Our results on serum glutamate were inconsistent with other results as we observed a significantly lower concentration of glutamate in our diseased groups compared to the healthy group. Glutamate may accelerate beta cell dysfunction caused by the hyperglycemic state [160]; this may be one reason why the level of glutamate increases in T2D.

As it was already proved, glycine is a potential biomarker and a predictor of prediabetes and T2D [73,76,152,158,159,161,162] and we could give further evidence to the already observed phenomenon [13,15,20,21,23–25].

Our result on the serum serine showed a significantly lower concentration in patients with obesity and T2D than in healthy subjects. Several studies reported similar results of decreased concentration of serine in patients with T2D compared to the control group [73,153] obese or overweight participants compared with those with normal weight [149] and patients with T2D in comparison with both healthy and obese groups [73]. Zhou *et al*. found that the concentration of serum serine was significantly lower in the case of patients with diabetes mellitus as compared to those with nondiabetes mellitus but not different between obese and lean groups [152]. In this regard, giving supplementary serine in diets for patients with T2D might help to decrease their blood glucose levels [152].

For citrulline, Zhou *et al*. and Yamaguchi *et al*. also found significantly higher concentrations of citrulline in the sera of patients with T2D as compared to controls [152,161]. However, our results were different from the above-mentioned ones, they aligned well with those of Okekunle *et al*. [73]. There was no significant difference between the obese and control groups regarding citrulline levels, but a statistically significant decrease was observed in the T2D group as compared to controls. According to the study by Takashina *et al*, there was no significant difference in the citrulline levels of obese and non-obese groups [158] while Newgard *et al*. found a significantly lower concentration of serum citrulline in obese individuals as compared with lean controls [76]. A decreased level of citrulline in T2D can be explained by the diminished bioavailability of nitric oxide in patients with diabetes [163]. Some

investigations reported that citrulline has a protective role against diabetes [164] and that the consumption of citrulline as a supplement may improve glucose homeostasis [165].

The significantly higher levels of isoleucine and leucine in patients with T2D (as compared to controls) are in good agreement with the data published in the scientific literature [153,161,162,166–169]. The consistent increase of BCAA during diabetes has been explained by numerous mechanisms, specifically a decline in insulin activity [161] and down-regulation of the BCAA-catabolism enzymes [76,170]. The degradation of BCAA starts in the muscle, whose metabolism is heavily altered in T2D. These amino acids were nominated as potential biomarkers for T2D [66,71,91,170]. Zhou *et al*. found a significantly lower concentration of leucine in the case of T2D as compared to the nondiabetes group, and a significantly higher concentration of leucine in the obese group compared to the lean group [152], but most of the findings suggest elevated BCAA levels in the blood of patients with T2D.

The decreased concentration of threonine in the T2D group as compared to the control group concurred with the observations of other scientists [73,153], however, Yamaguchi *et al*. had opposite findings [161].

As a result of biogenic amine analysis in the serum, two biogenic amines including ethanolamine and methylamine were present in higher amounts, however, only ethanolamine was analyzed since methylamine was not present in all samples. In terms of ethanolamine, we observed that its concentration was significantly lower in both cases of disease groups as compared to the control group. This result was in partial agreement with the literature, however, there were still inconsistencies in the findings of other studies. According to Fiehn *et al*., the concentration of ethanolamine is significantly lower in T2D individuals as compared to the non-diabetic group, whereas Calvani *et al*. and Zhou *et al*. found a significantly higher concentration of ethanolamine in the case of the T2D group as compared to the control group [152,157,168]. A recent study revealed ethanolamine as a potential biomarker and effective therapeutic agent for DR in patients with T2D [171]. We identified three other biogenic amines, ethylamine, putrescine, and serotonin which were present in the samples in trace amounts. Other biogenic amines, including histamine, cadaverine, tyramine, and phenethylamine were not detected in the sera. Our results suggest that polyamine catabolism may be associated with the regulation of energy and glucose metabolism [172].

According to the correlation analysis, we found 13 amino acids and 1 biogenic amine (out of the examined 33 compounds) to be correlated with the clinical parameters. These results support the outcomes of previous studies suggesting that amino acids can be crucial elements in evaluating the islet function as some of them were associated with insulin indicators such as

C peptide and HOMA-IR [74,155,158,173,174]. We found that the level of serine was negatively associated with insulin and C peptide concentrations, while the serum glycine level was negatively associated with insulin, C peptide, as well as HOMA-IR. In the case of the correlation between glycine and HOMA-IR, Badoud *et al*. found no significant correlation [74]. In contrast to this, Takashina *et al*. and Mohorko *et al*. found a negative correlation [155,158] which is in agreement with our results.

We found a positive correlation between isoleucine and HbA1c, similar to the results of previous studies [161,175,176]. However, contrary to other studies [74,153,168] we did not observe correlations between other BCAAs and HbA1C.

A positive correlation was observed between BMI and serum cysteine level. Elshorbagy *et al*. observed a similar phenomenon and showed that plasma cysteine was one of the strongest determinants of BMI [177,178]. Mohorko *et al*. revealed a significant strong association between BMI and cysteine [155], as well. In addition, the results of our correlation analysis corroborate with previous studies in terms of the negative correlation between BMI and amino acids, glycine, and serine [74,158,161].

WHR of the patients with obesity or T2D can be a reflection of the insulin resistance [179] and is positively correlated with the levels of BCAAs. Numerous data indicate that isoleucine, leucine, and valine are novel potential markers of insulin resistance during various pathological or non-pathological conditions [170]. The concentration of BCAAs was found to be associated with both hyperlipidemia and obesity-induced insulin resistance [170]. Our datas*et al*so supports this phenomenon. The positive correlation of isoleucine and leucine levels with triglyceride and the negative correlation of BCAAs with HDL were observed in our correlation analysis. These outcomes align with the clinical laboratory findings from another study that observed elevated triglyceride and LDL levels, as well as reduced HDL level in serum of patients with T2D [180]. Our study confirmed the positive correlation of triglyceride with levels of alanine and cysteine, as well as the negative correlation with glycine level.

There was observed a positive correlation between the concentration of cysteine and CRP level, while there was a negative correlation between the concentration of threonine and CRP. Studies have demonstrated that CRP can serve as an indicator of cardiovascular risk and may play an active role in the development of atherosclerosis [181]. Other research groups have observed a connection between CRP and cysteine levels similar to our findings [155]. There is currently no known evidence for the correlation between threonine and CRP.

Bembde *et al*. revealed a negative correlation of fibrinogen with histidine and threonine levels in diabetes [182], similar to our observation in the case of histidine, but not in case of

threonine. We observed a positive correlation between the concentrations of homocysteine and of citrulline and a negative correlation between the concentrations of ApoA1 and tyrosine, HDL, and phenylalanine. The correlation of HDL and phenylalanine [155] was demonstrated by other groups as well, but we could not find similar correlations between the concentration of homocysteine, ApoA1 and of amino acids in diabetes or obesity published in the scientific literature. At the moment, we can estimate the importance of these amino acids and biogenic amines in obesity and T2D without being able to give exact information.

The parameters reflecting kidney functions, such as ACR along with the GFR, negatively correlated with serum ethanolamine and glycine levels. We could notice that both metabolites were negatively correlated with BMI.

In our analysis we also wanted to go beyond the statistically significant changes in metabolites and correlation analysis data. We aimed to uncover potential disease-specific pathways and functions using a network model, to emphasize common pathological characteristics observed in both obese and T2D groups, as well as to identify new pathways of significance.

The applied network model allowed us to discern differences between the obese and T2D groups. In obesity, we observed alterations associated with energy generation, whereas in T2D, a profound involvement of NO synthesis and its relationship with insulin signaling and inflammation was the most prominent functions. The implication of enzymes involved in amino acid metabolism, particularly the metabolism of BCAA, glutamine, the urea cycle, and beta-oxidation, was characteristic of T2D.

Besides serum, tear is a biofluid with high potential as it can be collected non-invasively. Tear amino acids and other metabolites were examined in conjunction with T2D, and significant differences between healthy donors and patients with T2D could be observed [147]. Tear biomarkers helping to diagnose/predict diabetic retinopathy and to predict its progression would have high importance in the routine clinical diagnosis. The same analysis was carried out for tear as for the serum samples. 9 out of 10 examined biogenic amines were detected in low amounts in tears and only ethanolamine could be quantified, but no statistically significant differences between the tear samples originating from patients with obesity, T2D with no signs of diabetic retinopathy, and T2D with non-proliferative diabetic retinopathy was observed. The correlation analysis performed in the case of serum metabolites was also carried out in the case of tear metabolites. However, none of the analytes showed a statistically significant correlation. Our data indicate that very likely more patients should be recruited as this low sample size could not balance the high variance observed between the samples and hence, the tear metabolomics

could not distinguish between diabetic patients with or without DR in the current state. Previous tear proteomics studies carried out in our laboratory demonstrated statistically significant differences between healthy and diseased groups, especially the ones with advanced stages of DR [81]. Therefore, our results on tear amino acid and biogenic amine emphasize the need for a larger sample size encompassing both healthy control and patient groups to establish more robust conclusions.

6.2. Examination of amino acids and biogenic amines in grape-derived products

In order to do a comparative analysis of some grape-derived products, we aimed at analyzing their composition in terms of amino acids and biogenic amines. As a result, all the amino acids examined were detected and quantified in the selected Aszú and Furmint samples, while citrulline, glutamine, and tryptophan were not detectable in wine vinegar samples. In Essence, all amino acids - except tryptophan - were detected. The amino acid with the highest concentration was proline, followed by arginine, alanine, and leucine in all examined samples.

Our findings are in accordance with the results published in the scientific literature. According to Csomos *et al*. Tokaji Aszú and Szamorodni contain proline and arginine in the highest concentrations [183]. Similar trends were observed by other groups, however, the exact concentrations are different. In a study carried out by Kutlan *et al*. [184], the concentrations of arginine, glutamate, alanine, lysine, and aspartate were found to be high in a Hungarian white wine, Badacsonyi Szürkebarát, that originated from another geographical region of Hungary. This study focused on the optimization of the analytical method, the levels of citrulline, cysteine, histidine, ornithine, proline and taurine were not examined. Gomes-Alonso *et al*. [116] found approximately 10 times higher proline concentration in Spanish white wines than we did in the Tokaj wines. A similar result has been reported by Tuberoso *et al*. [115] in the sherry-like Italian wine Vernaccia di Oristano. They also found that besides proline, aspartate, glutamate, and glutamine were also abundant [115]. According to a study of Bouzas-Cid *et al*., proline was found to be the highest concentration amongst other amino acids in Portugal's Albarino white wine [185]. In some examined Greek white wines, arginine was present in the highest concentration [114,119]. Alanine, arginine, glycine, proline, and threonine were found to be the most abundant amino acids in a balsamic vinegar of Modena, Italy representing about 75% of the total amino acid content [107].

The type of microbes used for fermentation, the duration, and the conditions of fermentation shape the amino acid profile of wines [129]. Besides the fermentation, the grape

variety has a great influence on the amino acid content [119]. Wine vinegar is the product of a different type of fermentation, and we expected a marked difference between wine and wine vinegar samples. However, the concentrations of the amino acids in wine and wine vinegar samples were in the same range.

We successfully identified all the investigated biogenic amines, with the exception of histamine. Based on the existing scientific literature, which indicates the occasional presence of histamine in wine samples from various regions, we cannot dismiss the possibility that our pre-column derivatization followed by chromatography may not be a suitable technique for assessing histamine in wine and wine vinegar samples. At present, we have no sufficient information to determine whether histamine levels fell below our detection limit or if histamine was indeed absent from the samples. Moreover, we encountered challenges in distinguishing between the peaks of tryptamine and phenylethylamine, which limited our ability to provide separate quantification for these two biogenic amines. Among the biogenic amines analyzed, ethanolamine, ethylamine, and tyramine were found to have the highest concentration in the samples, and these findings were consistent with previous studies [131,183,186,187]. However, in all cases, the levels of these amines remained below the toxicity thresholds established by the European Food Safety Authority (EFSA) for fermented food [188].

According to Csomos *et al.* in Aszú wine histamine, putrescine and tyramine were the dominant biogenic amines, while in Szamorodni wines putrescine and tyramine [183]. The biogenic amine ethanolamine was found to have the highest concentration in Hungarian white wine, Badacsonyi Szürkebarát, and Italian white wine [184,187]. Ethanolamine was detected in the highest concentration in chardonnay, Passerina, Pecorino, and Trebbiano Italian white wines, followed by putrescine [131]. Our results are in accordance with those of Manetta *et al*., who identified ethanolamine, ethylamine and putrescine as the most abundant biogenic amines in the analyzed white and red wines [186]. In Sauvignon Blanc, a Chilean reserve varietal wine, putrescine was the biogenic amine with the highest concentration followed by histamine and tyramine [133]. In the case of Italian sherry-like wine, Vernaccia di Oristano [115], Italian white wines [130], Spanish white wine [189], and Greek white wines [190] similar results were found. In our study, putrescine was not an abundant biogenic amine and when compared to the other samples, showed a significantly higher concentration in Furmint.

In the study done by Torre *et al*. in Sicilian white wines the concentrations of cadaverine, histamine, and tyramine were near the detection limit and phenylethylamine tryptamine were not detected [191]. These results were similar to the ones obtained by Manetta *et al*. and Bover-Cid *et al*. [186,192], highlighting the fact that variations in the amount and detection of the

analytes depend on the type of the examined wine and the applied experimental setup. In our study, in all but one Essence sample serotonin was present in low concentration. In most of the reviewed experimental setups, serotonin was not measured at all. Those who measured serotonin found its concentration low, such as in some Spanish [192] and Italian white wines [130].

In wine vinegar, we have not detected histamine and serotonin. In our samples, tyramine had the highest concentration followed by ethanolamine. The concentration of cadaverine, methylamine, putrescine, and tyramine were the lowest in wine vinegar among all our examined samples. Kutlan *et al*. identified ethylamine as a biogenic amine having the highest concentration in Hungarian wine vinegar [184]. The biogenic amines found in white wine vinegar from Spain were cadaverine, putrescine and spermine [193]. The discrepancy among the results is even higher in the case of biogenic amines than in the case of amino acids. There are remarkable differences in the methods utilized for the detection of biogenic amines and also in the range of the analytes examined by the different groups.

Given the variations in grape type and fermentation conditions among the three examined wine types, our results emphasize the significance of grape variety and reveal distinct patterns in how *Acetobacter* species utilize amino acids during acetic fermentation, as opposed to *Saccharomyces* species during alcoholic fermentation.

As we mentioned, Aszú wine is produced from the Furmint wine poured over botrytized grape berries. We hypothesized that the amino acid and biogenic amine content of Furmint and Aszú might reflect the effect of botrytized grapes. In order to reveal these differences, we carried out a pair-wise analysis by comparing Aszú and Furmint wines originating from the same vinery and year. As we expected, we found distinct patterns of differences: the direction of changes in the case of 11 amino acids and of 3 biogenic amines was consistent and very likely reflected the effect of the presence of botrytized grape. Our data indicate that the botrytized grapes in Aszú wine have a substantial influence on the concentrations of citrulline, cysteine, glutamate, glutamine, glycine, histidine, lysine, methionine, serine, threonine, tryptophan, ethanolamine, ethylamine and putrescine as compared to Furmint wine obtained from the same winery and year. These findings highlight the distinctive characteristics of Aszú and Furmint wines and indicate a significant role of botrytized grapes in modifying the concentration of these metabolites.

In summary, our findings propose valuable insights into the amino acids and biogenic amine composition of various wine and wine vinegar samples and may highlight their utility as potential functional food.

SUMMARY

Diabetes and obesity are pathological conditions affecting millions of people worldwide. Examination of metabolites and of their relation with other clinical parameters can give valuable information on the pathophysiological changes and eventually can lead to identification of new potential biomarkers allowing for a better patient stratification and prediction of complications. We determined the changes in the levels of amino acids and biogenic amines in obesity and T2D as compared to healthy controls, and we have successfully proved already known and demonstrated some new, previously unknown correlations between the levels of amino acids and clinical laboratory parameters and patients data, respectively. The applied network model also provided useful information about the pathways and protein associations that are characteristic to obesity and/or T2D. We acknowledge the need for further research to validate the emerging concepts and gain a deeper understanding of the complex metabolic dysregulation associated with obesity-induced insulin resistance and T2D. Besides serum, tears was also examined, but due to the high variance in the data and the relatively low sample number available, no statistical significances were established between the studied groups. This points out to one of the main limitations of our study, namely the requirement for more donors to enable a better patient stratification and the utilization of tears for diagnostic purposes. Despite these limitations, our results offer valuable information that can serve as potential targets for mechanistic studies aimed at developing future therapies for insulin resistance in advanced obesity and T2D.

In our next study we examined the amino acid and biogenic amine concentrations in various grape-derived beverages. The level of amino acids and biogenic amines in Furmint, Aszú, and Essence wines, as well as wine vinegar from the Tokaj wine region was characteristic to the studied beverage, reflecting their different production process. By comparing Aszú-Furmint wine pairs from the same winery and year, we identified some amino acids and biogenic amines sensitive to the presence of botrytized grapes in Aszú. Our experimental setup can provide a relatively fast and easy-to-implement analytical approach for the quality control and nutraceutical examination of wine and wine vinegar.

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