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Characteristics of amino acid profile and incretin hormones in patients with gallstone disease - a pilot study

Short title: Amino acids in patients with gallstone disease

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Conflict of interest: none declared.

What's new?

Branched-chain amino acids, which profile is altered in gallstone disease, may link gallstone disease with insulin resistance, type 2 diabetes mellitus and cardiometabolic syndrome.

Abstract

Introduction: Gallstone disease is associated with insulin resistance, type 2 diabetes mellitus and increased risk of incident ischemic heart disease. It is known that branched-chain amino acids (BCAAs) profile is altered in cardiac diseases and in metabolic diseases, such as diabetes, obesity. The role of BCAAs in gallstone disease is still not known.

Objectives: The aim of this study was to evaluate the concentration of essential amino acids and incretin hormones in patients with cholecystolithiasis.

Patients and methods: The study included 31 patients with cholecystolithiasis and 25 gallstone-free controls. Free exogenous and endogenous amino acids, bile acids, glucagon-like peptide 1 (GLP-1), glucose-dependent insulintropic polypeptide (GIP), ghrelin, C-peptide and insulin were measured in the fasting state and one hour after consumption of a 300-kcal mixed meal.

Results: The mean fasting and postprandial levels of BCAAs: valine, isoleucine, leucine and lysine were significantly higher in the study group than in the controls ($p=0.005$ - <0.001). The percentage increase in concentrations of amino acids after a meal were similar in both groups of patients. The mean fasting concentrations of C-peptide and GLP-1 were higher in the study group than in the control group ($p=0.004$ and $p=0.03$, respectively), and the median postprandial concentration of C-peptide was higher in the study group as compared to the control group ($p=0.03$).

Conclusions: Diabetes mellitus, coronary heart disease and gallstone disease might have common genetic and environmental antecedents. However, higher plasma levels of BCAAs

observed in patients with gallstone disease may be partly responsible for metabolic complications observed in these patients.

Key Words: amino acids; gallstone disease; incretin hormones

Introduction

Gallstone disease (GD) constitutes a significant health problem in developed societies, affecting 5% to 25% of the adult population [1,2]. Epidemiological studies have indicated a large number of risk factors for cholesterol stones. Physical inactivity and overnutrition are known risk factors for obesity and metabolic syndrome [3]. Increasing prevalence of obesity, diabetes mellitus and related adipose tissue dysfunction will be relevant trigger for cardiovascular diseases [4]. During excessive intake of food the synthesis of whole-body cholesterol is promoted, and its excess is eliminated with the bile. Thus, risk for gallstones is greatly increased [5]. Moreover, insulin resistance and type 2 diabetes mellitus act as independent factors associated with gallstone disease [6,7]. Hyperinsulinemia is related to increased hepatic cholesterol uptake, biliary secretion and hyosecretion of biliary bile acids [3]. In contrast, gallstone disease is associated with increased risk of incident ischemic heart disease (IHD), independent of traditional risk factors [8]. In the German population, persons with gallstones were at increased risk of myocardial infarction and stroke; however, these persons did not experience a lowered cardiovascular risk after gallbladder removal [9]. It has also been reported that alterations in essential amino acids profiles in the blood are associated with cardiovascular disease (CVD) [10].

Essential amino acids necessary for proper human development are not synthesized in the body and must be ingested with food (exogenous essential amino acids such as isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr),

tryptophan (Trp), valine (Val), and histidine (His)). Among these essential amino acids, branched-chain amino acids (BCAAs: Val, Leu, and Ileu) are abundant in food, accounting for approximately 20% of total protein intake [11]. Despite clinical evidence suggesting that the supplementation of essential amino acids or BCAAs has beneficial effects on body weight, body fat, lean body mass, and insulin sensitivity, the increased levels of BCAAs may cause insulin resistance and diabetes mellitus type 2 [11,12]. Higher concentrations of BCAAs in plasma are associated with increased risk of CVD, especially stroke [13]. In patients after myocardial infarction, cardiac BCAAs catabolism is impaired, resulting in myocardial BCAAs accumulation. Then, BCAAs activate myocardial mammalian target of rapamycin signaling and subsequently contribute to cardiac dysfunction and remodeling following myocardial infarction [14]. The role of BCAAs in gallstone disease is not known, and no data exist linking BCAAs with the risk of this disease.

Gastrointestinal hormones (glucagon-like peptide-1: GLP-1; glucose-dependent insulinotropic polypeptide: GIP), known as incretins, stimulate insulin secretion at physiological concentrations. The incretin activity is linked to the gastrointestinal processing of ordinary meals [15]. New drugs have been developed for diabetes therapy. Incretin-based drugs, such as glucagon-like peptide 1 (GLP-1) analogues, are relatively new antidiabetic therapies recommended for patients with type 2 diabetes mellitus [16,17]. GLP-1 analogues induce activation of the GLP-1 receptor, which stimulates insulin secretion in a glucose-dependent fashion while also inhibiting secretion of glucagon. The use of GLP-1 analogs was associated with an increased risk of bile duct and gallbladder disease [16].

The aim of this study was to evaluate the concentration of essential amino acids and incretin hormones in patients with cholecystolithiasis.

Patients and Methods

Thirty-one consecutive patients with symptomatic gallstone disease scheduled for elective surgery (laparoscopic cholecystectomy) were selected for the study group (21 females, mean age (SD): 56.2 (12.9) years; 10 males, mean age (SD): 51.5 (12.7) years). Gallstones were visualized in the gallbladder on ultrasound examination before the operation. Patients presented with mild, recurrent episodes of biliary colic. None of these patients had associated choledocholithiasis or acute cholecystitis. The control group consisted of twenty-five consecutive patients (18 females, mean age (SD): 47.0 (15.7) years; 7 males, mean age (SD): 67.1 (7.5) years) who were treated for hernias or had colonoscopy examination and had no pre- or intraoperative signs of cholelithiasis or jaundice. All patients were surgically treated in the Department of General and Oncological Surgery (St. John Grande Hospital in Krakow) from 2017 to 2018. The characteristics of the study population are reported in Table 1. White blood cell count, glucose, thyroid stimulating hormone (TSH) and free thyroxine (FT4) were comparable between the study and control groups, while BMI was significantly higher in the study group.

From all patients, fasting venous blood samples and venous blood samples one hour after the consumption of a 300 kcal-mixed meal, which consisted of 24% protein, 41% carbohydrates and 35% fat (Nutridrink protein, 125 ml, Nutricia), were collected separately on lithium heparin and on EDTA with aprotinin. Blood was additionally collected to tubes containing a clot activator in order to obtain the serum for further analyses. The blood was centrifuged for 10 minutes at $1200 \times g$, and the plasma and serum were collected and kept at -70°C until analysis. Quantitative determination of free amino acids in the plasma was performed by the Pico-Tag method (Waters, USA). Samples were analyzed by a high-performance liquid chromatography with UV-VIS detection (Waters, USA) as previously described by Bugajska et al. [18]. The essential amino acids: valine (Val), isoleucine (Ile), leucine (Leu), threonine (Thr), methionine (Met), phenylalanine (Phe), lysine (Lys), tryptophan (Trp), histidine (His)

and nonessential amino acids: arginine (Arg), tyrosine (Tyr), aspartic acid (Asp), glutamic acid (Glu), hydroxyproline (Hypro), serine (Ser), asparagine (Asn), glycine (Gly), glutamine (Gln), taurine (Tau), citrulline (Cit), alanine (Ala), proline (Pro), α -aminobutyric acid (AAB), ornithine (Orn) were determined.

The total bile acid (TBA) content was measured using an enzymatic colorimetric method (bile acid (TBA), Randox Laboratories Ltd, United Kingdom). Plasma glucagon-like peptide 1 (GLP-1), plasma ghrelin, and glucose-dependent insulinotropic polypeptide (GIP) were assayed using an ELISA kit (Millipore, USA; Millipore, USA; Immuno-Biological Laboratories Co., Ltd. Japan; respectively). C-peptide concentrations were measured in serum samples with RIA (radioimmunoassay) method (DIAsource C-PEP II- RIA-CT Kit, Belgium). Insulin, TSH, and FT4 concentrations were determined using the immunochemiluminescence method, with the ADVIA Centaur® XP equipment.

The study was approved by the Jagiellonian University Bioethics Committee (Protocol No. 1072.6120.44.2017). All patients who participated in the study signed a free and informed consent form and were fully informed on all aspects related to the research. All methods performed in the study were conducted following all ethical and legal regulations.

Statistical analysis

Descriptive statistics (mean values, SD, medians, quartiles Q1-Q3) were used in the statistical assessment of the obtained results. Calculations were performed using Statistica software version 10 (StatSoft) and Microsoft Office Excel 2003. Shapiro-Wilk test were used to test the normality of variables. To compare parameters between the study group and the control group for normally distributed continuous variable the student's t-test was used, in case of not-normal distribution, the Mann-Whitney U test was used. The level of significance was set at $p < 0.05$.

Results

The concentrations of plasma essential amino acids from patients with cholelithiasis and from control subject are presented in Figure 1. The fasting and postprandial values of valine, isoleucine, leucine (branched-chain amino acids) and lysine were significantly higher in the study group than in the control ($p=0.005 - < 0.001$).

Table 2 presents the fasting and postprandial values of total bile acids, hormones and nonessential amino acids concentrations obtained in the study group and in the control group. The fasting concentrations of tyrosine, glutamic acid, alanine, proline, and ornithine were significantly higher in the study group than in the control ($p=0.009$, $p=0.02$, $p=0.03$, $p=0.02$, and $p=0.03$, respectively), whereas the postprandial concentrations of arginine, tyrosine, proline, α -aminobutyric acid and ornithine were significantly higher in the study group than in the control ($p=0.01$, $p=0.006$, $p=0.02$, $p=0.04$ and $p=0.02$, respectively). The values of other amino acids were similar in both studied groups. The percentage increase in concentrations of amino acids after a meal were the same in both studied groups.

The fasting concentrations of C-peptide and GLP 1 were higher in the study group than in the control group ($p=0.004$ and $p=0.03$, respectively), and the postprandial concentration of C-peptide was higher in the study group than in the control group ($p=0.03$). There were no significant differences for total bile acids, insulin, ghrelin and GIP between the two groups.

Discussion

The prevalence of gallstone disease is significantly higher in diabetic patients than in members of the general population with comparable characteristics (MICOL study) (24.8% and 13.8%, respectively) [19,20]. The results of Weng et al. meta-analysis supported the viewpoint that diabetes mellitus increases the risk of gallstone disease [21]. Association between GD and diabetes mellitus is frequently linked to obesity [21]. Furthermore, the association between high BMI and gallstone disease might be explained by insulin resistance. Gallbladder dysmotility might, thereby, be the mechanism of gallstone formation in insulin

resistance [6]. Caroli et al.[22] found a positive association between higher C-peptide levels and cholelithiasis in diabetic patients. Würtz et al. [23] have shown that circulating branched-chain amino acids (isoleucine, leucine, valine) and aromatic amino acids (phenylalanine, tyrosine) from fasting serum are predictors of insulin resistance, but not glycemia, in young adults at a 6-year follow-up. BCAAs likely promote insulin resistance through activation of mammalian target of rapamycin complex 1 [24].

In the present study, the mean/median values of branched-chain amino acids, tyrosine, C-peptide and insulin were higher in patients with cholelithiasis than in control subjects; however, for insulin, the difference was not statistically significant. These observations agree with the results obtained by Mendez-Sanchez et al.[25], who analyzed the serum concentrations of insulin in patients with cholecystolithiasis and noticed a greater tendency towards insulin resistance in that group than in controls.

Altered metabolism of branched-chain amino acids (BCAAs) and their subsequent accumulation in the blood may precede the development of insulin resistance and clinical manifestation of cardiometabolic diseases [26]. Increased BCAAs levels are independent risk factors of metabolic syndrome and cardiovascular disease in middle-aged and elderly Chinese populations [27]. Shah et al. [28] used quantitative mass spectrometry to measure metabolomic profiles in 117 individuals within eight multiplex families from the Genecard study of premature coronary artery disease (CAD), and results revealed that some AAs (arginine, ornithine, alanine, proline, leucine, isoleucine, valine, glutamine, phenylalanine and glycine) were highly heritable and could be used to identify families with premature coronary artery disease. Mangge et al. [29] investigated the possibility of employing branched-chain amino acids (BCAAs) to identify an increased CVD risk. They observed significantly higher serum concentrations of valine, isoleucine and leucine in the obese ($BMI > 30$, kg/m^2) and overweight ($BMI = 25,1-30$, kg/m^2) cardiometabolically abnormal subjects than in

cardiometabolically healthy subjects. The aromatic AAs, such as phenylalanine and tyrosine, were significantly increased in the overweight and obese cardiometabolically abnormal subjects compared to cardiometabolically healthy subjects. Ornithine was significantly increased in the overweight cardiometabolically abnormal subjects compared to cardiometabolically healthy subjects [29]. Similar results were obtained in the present study, where these amino acids were more abundant in the study group than in the control group. Mendez-Sanchez et al. [25] found a statistically significant negative association of ghrelin serum levels and the prevalence of gallstone disease using a multivariate model in a logistic regression analysis, but the median of serum ghrelin values did not show a differences between patients and controls. Similar results were obtained in the present study: the mean fasting and postprandial concentrations of ghrelin were lower in the study group than in the control group, but this difference was not statistically significant.

The BCAAs given orally stimulate secretion of insulin, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [30,3]. Our study revealed statistically significantly higher fasting concentrations of GLP-1 in patients with cholecystolithiasis than in controls ($p=0.03$) and higher GLP-1 postprandial concentrations in the study group than in the control group, but these differences were not statistically significant ($p=0.07$). In patients with gallstone disease, a higher concentration of GLP-1 might be caused by higher concentrations of BCAAs. Similar results were obtained for GIP, but these differences were not statistically significant. To our knowledge, this is the first study comparing AAs profile and incretin concentrations in plasma in gallstone patients.

In the present study, the mean fasting and postprandial values of BCAAs were significantly higher in the study group than in the control group, and the percentage increase in the concentrations of amino acids after a meal were the same in both groups. These results may suggest that high baseline concentrations of BCAAs might be biomarkers of the underlying

metabolic dysfunctions (metabolic fingerprint) rather than the result of food ingestion. These observations confirm the results obtained by Ruiz-Canela et al. [13]. They showed that the Mediterranean diet has a negligible effect on 1-year changes in BCAAs. It likely exerts its cardioprotective effects via alternative pathophysiological processes.

Taking into account the results of the present study, we hypothesized the following:

- diabetes mellitus, coronary disease and gallstone disease might have common genetic and environmental antecedents. It has been already speculated by Stern [32] that both diabetes and cardiovascular disease share common antecedents rather than one being a complication of the other ("common soil hypothesis").

- complications observed in gallstone disease are not probably related to the disease as such, but higher BCAAs levels might be the cause of diabetes mellitus and cardiovascular disease.

A large multicenter study is necessary to evaluate the relationship between plasma amino acids and diabetes, gallstone disease, and cardiovascular disease.

Author contributions:

Jolanta Bugajska: planning and conducting the study, collecting the data, interpreting data, drafting the manuscript, and critical revisions of the manuscript. Katarzyna Gotfryd-Bugajska: conducting the study, collecting the data, and critical revisions of the manuscript. Mirosław Szura: planning the study interpreting data, and critical revisions of the manuscript. Joanna Berska: collecting the data, interpreting data, and critical revisions of the manuscript. Artur Pasternak: planning the study, interpreting data, and critical revisions of the manuscript. Krystyna Sztefko: planning and conducting the study, interpreting data, and critical revisions of the manuscript. All authors approved the final manuscript.

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Table 1. Characteristics of the study populations.

	Study group n=31	Control group n=25	P
	Mean (SD) or Median (Interquartile range)		
Body mass index, kg/m²	29.7 (3.39)	26.3 (3.28)	<0.001
White blood cell count, 10 ⁹ cells/l	6.57 (1.55)	6.57 (1.53)	0.99
Fasting blood glucose, mmol/l	5.56 (0.56)	5.52 (0.66)	0.84
TSH, mIU/l	1.51 (0.81-4,18)	1.52 (1.03-1.88)	0.63
FT4, pmol/l	16.9 (1.9)	17.2(2.2)	0.55

FT4 - free thyroxine; TSH - thyroid stimulating hormone

Table 2. Fasting and postprandial plasma mean concentrations of total bile acids, hormones and nonessential amino acids (SD) in the control group and in the study group.

	Fasting			Postprandial		
	Study group n=31	Control group n=25	P	Study group n=31	Control group n=25	P
	Mean (SD) or Median (Interquartile range)			Mean (SD) or Median (Interquartile range)		
Total bile acids, $\mu\text{mol/l}$	1.47 (0.81-2.73)	1.46 (1.03-2.64)	0.39	2.92 (1.61-4.82)	2.5 (1.54-3.18)	0.37
Hormones						
Insulin, $\mu\text{IU/ml}$	11.58 (6.22)	9.11 (3.73)	0.16	66.09 (39.35)	44.9 (21.2)	0.06
C-Peptide, ng/ml	2.99 (1.13-6.16)	2.29 (1.49-3.92)	0.004	7.44 (5.42-8.75)	5.3 (4.87-6.7)	0.03
Ghrelin, pg/ml	643.6 (233.5)	691.1 (285.3)	0.60	598.4 (195.7)	620.2 (263.4)	0.78
GLP 1, pmol/l	38.7 (16.5)	27.1 (7.64)	0.03	49.7 (18.2)	39.0 (12.3)	0.07
GIP, pmol/l	30.8 (23.9-36.3)	27.8 (17.6-36.0)	0.19	205.7 (169.6-208.7)	171.9 (137-208)	0.32
Nonessential amino acids, $\mu\text{mol/l}$						
Arginine	114.4 (98.6-144.2)	112.8 (91.6-120.7)	0.37	149.3 (141.2-178.9)	128.1 (109.5-147)	0.01
Tyrosine	69.5 (60-72.7)	60.8 (53.9-71.1)	0.009	100.2 (19.9)	84.4 (21.6)	0.006
Aspartic acid	6.2 (2.3-11.9)	4.3 (1.75-8.4)	0.14	5.8 (2.2-13.3)	3.2 (2-9.7)	0.2
Glutamic acid	120 (61.1-209.3)	89.4 (49.9-162.4)	0.02	141.8 (76.3-263.6)	92.2 (54.5-183.3)	0.07
Hydroxyproline	8.3 (6.6-13.3)	8.1 (6.35-11.4)	0.14	9 (7.9-14.7)	8.5 (7.2-11.9)	0.31
Serine	110.4 (27.5)	117.5 (28.0)	0.34	135.1 (33.0)	153.2 (36.5)	0.06
Asparagine	59.9 (47.7-68.9)	60.2 (48.9-68.9)	0.84	82.0 (73.2-92.0)	81.1 (74.0-90.9)	0.81
Glycine	212.6 (60.5)	213.2 (64.0)	0.97	215.6 (58.3)	224.3 (67.6)	0.61
Glutamine	701.1 (146.6)	657.0 (123.9)	0.24	772.0 (150.3)	753.7 (168.3)	0.67
Taurine	41.45 (11.8)	39.84 (8.22)	0.57	43.3 (11.6)	42.0 (8.8)	0.67
Citrulline	31.47 (12.28)	33.02 (11.75)	0.64	32.7 (13.2)	33.12 (10.5)	0.91
Alanine	573.9 (142.5)	478.8 (182.2)	0.03	722.6 (164.4)	636.1 (206.9)	0.09
Proline	262.9	214.8	0.02	458.4	323.6	0.02

	(197.2-377.8)	(167.3-323.6)		(354.9-512.4)	(260.4-401.8)	
α-Aminobutyric acid	40.5 (27-58.9)	32.8 (25.3-47.5)	0.08	55.3 (36.1-80.2)	36 (27.5-60.1)	0.04
Ornithine	48.3 (42.6-70.3)	45.6 (38-54.8)	0.03	66.7 (54.0-80.1)	55.6 (44.4-73.0)	0.02

GIP - glucose-dependent insulinotropic polypeptide; GLP-1 - glucagon-like peptide-1

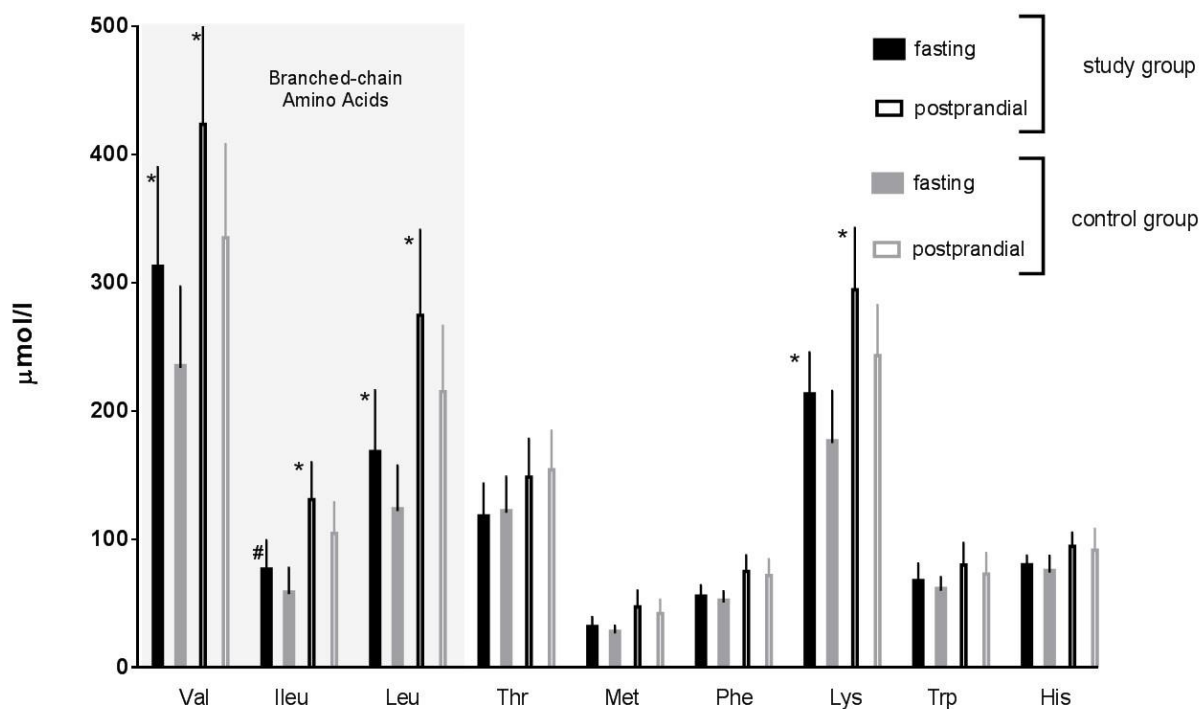


Figure 1. Concentrations of essential amino acids (mean (SD)) in plasma from patients with cholelithiasis and from control subjects.

* $p < 0.001$

$p = 0.005$

compared to the control group