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Running title**: GSG-index: a new marker of severe liver disease**

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Abstract (n=269)

Plasma concentrations of amino acids (AA), in particular branched chain (BCAA), are often found increased in non-alcoholic fatty liver disease (NAFLD). However, if this is due to increased muscular protein catabolism, obesity and/or increased insulin resistance (IR) or impaired tissue metabolism is not known. Thus, we evaluated a) if subjects with NAFLD non-obese (NAFLD-NO), compared to obese (NAFLD-Ob) display altered plasma AA compared to controls (CT); b) if AA concentrations are associated to IR and liver histology. Also glutamic acid, serine and glycine concentrations were previously found altered in NAFLD. Since these AA are involved in glutathione synthesis we hypothesized they might be related to the severity of NAFLD.

In 44 non-diabetic NAFLD subjects with liver biopsy, (29 NAFLD-NO and 15 NAFLD-Ob) and 20 non-obese CT we measured AA profile by GCMS, HOMA-IR, hepatic IR [Hep-IR=Endogenous Glucose Production x Insulin] and the new GSG-index [glutamate/(serine+glycine)] and tested if they were associated with liver histology.

Most AA were increased only in NAFLD-Ob. Only alanine, glutamate, isoleucine and valine, but not leucine, were increased in NAFLD-NO compared to CT. Glutamate, tyrosine and GSG-index were correlated with Hep-IR. GSG-index correlated with liver enzymes, in particular GGT (R = 0.70), independently of BMI. Ballooning and/or inflammation at liver biopsy were associated with increased plasma BCAA and Aromatic-AA, and mildly with GSG-index, while only the new GSG-index was able to discriminate fibrosis F3-4 vs. F0-2 in this cohort.

Conclusions: Increased plasma AA concentrations were observed mainly in obese NAFLD, likely as a consequence of increased IR and protein catabolism. The GSG-index is a possible new marker of severity of liver disease independently of BMI.

Introduction

The prevalence and incidence of non alcoholic fatty liver disease (NAFLD) is increasing (1). The mechanisms that favor the development of simple steatosis and the following transition to inflammation, ballooning and fibrosis (i.e., non alcoholic steatohepatitis, NASH) are still not well understood. In addition, it is necessary to identify novel biomarkers of altered mechanisms that can follow the progress of the disease. Metabolomics, has provided insights into mechanisms underlying development of several diseases as insulin resistance, as T2DM and NAFLD, and also discovered potential biomarkers of NAFLD and its severity (2-7). Among metabolites increased amino acid (AA) concentrations, especially branched chain amino acids (BCAA) have been associated with an increased risk of metabolic disease, including T2DM and NAFLD (8-10). However, it has been questioned if amino acids are really causally linked to type 2 diabetes and other metabolic diseases, or if increased concentrations were due to prevailing insulin resistance (IR) or possibly impaired tissue metabolism (9, 11, 12). Fasting plasma levels of branched chain amino acids (BCAA, i.e., leucine, isoleucine and valine) are often found increased and associated with peripheral insulin resistance (IR) (6, 8). Plasma concentration of aromatic amino acids (AAA, e.g., tyrosine and phenylalanine) were also found increased with severity of liver diseases (13). These results are not surprising since the liver is the site of protein and amino acid metabolism (both synthesis and catabolism) (**Figure 1**).

Patients with NAFLD have increased both peripheral and hepatic insulin resistance (Hep-IR) (14) although the pathophysiological mechanisms for increased Hep-IR in NAFLD are still not known. Amino acids might mediate the activation of several hepatic metabolic pathways involved in insulin signaling and glucose regulation (11). For example it has been hypothesized that BCAA might increase hepatic IR through mTOR signaling (8, 10, 15). However, since during fasting most of circulating amino acids derive from muscle catabolism (16), that is increased in IR states, it is likely that alteration in amino acid concentrations reflects IR rather than presence of NAFLD. Most of the previous studies involved mainly obese insulin resistant subjects where amino acid catabolism is upregulated and thus it is not easy to establish if increased fasting concentrations of amino acids are related to NAFLD or to peripheral insulin resistance. The studies in animals (e.g., (15)) are also non conclusive since they compare age matched animals treated with high fat diet (vs chow diet,) without accounting for the increase in insulin resistance. Beside BCAA and AAA other amino acids like glutamate, glutamine, alanine and aspartate have been found positively

associated with increased cardiometabolic risk and in particular with hepatic insulin resistance (12, 17-19), while glycine and serine were found decreased in metabolic diseases including NAFLD (19-23). In Table 3 we have reported the most relevant papers that show alterations in AA metabolism in obesity/NAFLD**.** Thus, the first aim of our study was to evaluate if plasma amino acids concentrations were increased/decreased similarly in obese and non obese subjects with NAFLD and their association with the degree of fasting hepatic and peripheral insulin resistance. As second aim we evaluated if alterations in plasma amino acid concentrations (such as BCAA, AAA, glutamate, glycine and serine), demonstrated to alter cell function or to be involved in mitochondrial function and oxidative stress, might reflect hepatic inflammation and/or fibrosis.

2. Methods

We have studied 44 non-diabetic patients with biopsy-proven NAFLD and 20 healthy controls (CT), **Table 1**. All controls had normal liver enzymes, normal liver at US-scan and low probability of hepatic fat according to non-invasive indexes (Fatty liver Index < 20 and NAFLD liver fat Score > 1.257). NAFLD patients were divided in non obese (NAFLD-NO, BMI<30 kg/m2, n=29) and obese (NAFLD-Ob, BMI ≥30 kg/m2, n=15).

Liver biopsy was scored according to Kleiner et al (24). The sum of grading for steatosis, lobular inflammation and hepatocellular ballooning was used to calculate the NAFLD activity score (NAS) from 0 to 8. Fibrosis was staged 0 to 4 and classified as absent (0), mild (1-2) and severe (3-4). NASH was defined by the local pathologist according to the joint presence of steatosis, hepatocyte ballooning and lobular inflammation with or without fibrosis.

In each subjects we collected a blood sample after an overnight fast to measure liver enzymes, lipid profile, concentrations of glucose, free fatty acid, insulin, amino acids.

All subjects participated in the study after signing an informed consent.

All the other investigations were carried out during regular follow-up of NAFLD patients, according to specific protocols, and all patients had given their consent for including their personal data in the database. The study was approved by the ethics committee of the University Hospital San Giovanni Battista of Torino and was in accordance with the Helsinki Declaration.

*Measurements and calculations***.**

Plasma concentrations of lipids, glucose, insulin, liver enzymes and lipid profile were measured as previously reported (25, 26). Amino acid plasma profile was evaluated on fasting samples by GCMS (Agilent technology GC7890-MS5975). Briefly, 80uL of EDTA plasma sample was deproteinized with 1 ml methanol, and subsequently purified by ion exchange columns and derivatized using N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide, Sigma, USA). Plasma amino acid concentrations were determined using as internal standard a mix of ^{13}C labeled amino acids (Celtone, CIL Cambridge MA, USA) that contains uniformly labelled phenylalanine, tyrosine, leucine, isoleucine, valine; alanine, lysine, hystidine, proline, threonine, glutamate, serine and glycine. Our method provides a quantitative analysis of plasma amino acid profile, compared with the usual metabolomics methods that are semiquantitative. Using this approach we were able to quantify concentrations of aromatic amino acids (AAA), phenylalanine and tyrosine; branched chain amino acids (BCAA) leucine, isoleucine and valine; alanine, lysine, histidine, proline, threonine, glutamate, serine and glycine, reported in **Table 2**.

We calculated a new index based calculated by the ratio of glutamate to serine plus glycine concentrations [Glutamate/(Serine+Glycine)], named the GSG-index (from the initials of the AA). The concentrations of these amino acids were previously found altered in metabolic diseases including NAFLD (**Table 3**); in particular glutamate concentrations were increased while glycine and serine were decreased (19-23). These amino acids are in some way involved in the synthesis of GSH (glutamic acid and glycine directly, while serine indirectly since it is a precursor of glycine) and of lipids associated to lipotoxicity (i.e., ceramides) (**Figure 1**). We therefore tested if GSG index was associated with alterations in liver histology.

Insulin resistance

We have also quantified degree of insulin resistance in liver and muscle (denominated peripheral insulin resistance).

Hepatic insulin resistance (Hepatic-IR) that reflects the inability of insulin to suppress fasting endogenous glucose production (EGP) was calculated as Hep-IR =(EGP x fasting insulin) (27). EGP was measured by the kinetics of 6,6-D2-glucose infused for 2 hours during fasting state as previously reported (25) in all subjects with NAFLD and in 8 CT. Peripheral insulin sensitivity

was calculated as fasting glucose rate of disappearance (Rd) measured by the kinetics of 6,6-D2-glucose normalized by fasting insulin (Rd/I) (25).

HOMA-IR, calculated as (Glucose x Insulin)/22.5, reflects both hepatic and peripheral IR as previously described (26, 28).

We tested if the degree of IR was different in obese vs non obese NAFLD and if the concentrations of AA were altered differently in obese vs non obese NAFLD compared to CT.

Statistical analysis.

The variables were expressed as mean +/- standard error (SE). The difference between controls and NAFLD subjects was calculated using the parametric test (ANOVA, or t-test) when normally distributed or using nonparametric test Mann-Whitney and Kruskal Wallis when variables were non normally distributed (StatView and JMP, SAS Institute Inc). For correlation analysis we used Pearson's correlation coefficient for normally distributed variables and Spearman's rank correlation for non-normally distributed variables. For multivariable correlation analysis we reported partial correlation coefficient. For the analysis of the association between amino acid concentrations and liver histology (**Figure 3-5**), AA concentrations in NAFLD were divided by mean values observed in CT and then Log2 transformed to evaluate the fold changes compared to CT. Statistical significance was tested using Wilcoxon signed rank test. We used multivariable analysis to evaluate which among BCAA, AAA and GSG index was independently associated with indexes of insulin resistance. We used logistic regression analysis to evaluate which among BCAA, AAA and GSG index (used simultaneously as independent variables) had the highest odd ratio (OR) for ballooning grade 2, inflammation or liver fibrosis (grade 3-4), also accounting for BMI. All non normally distributed variables were $\log_e$-transformed before the analysis.

Results

Clinical characteristics of study subjects.

Table 1 summarizes the anthropometric, clinical and biochemical parameters of the study population (NAFLD and controls, CT). Overall, BMI ranged from 18 to 36 kg/m^2. However, the great majority of NAFLD subjects (29 over 44) had a BMI<30 kg/m^2 and the BMI of NAFLD-NO was similar to CT (25.6±0.5 vs 24.1±0.8 kg/m2, NAFLD-NO vs CT, p=ns). As expected, compared to CT, subjects with NAFLD had higher concentrations of liver enzymes (ALT, GGT, AST) (**Table 1**,

all p <0.0001). Insulin resistance was higher in NAFLD-Ob compared to NAFLD-NO and to CT both when calculated as HOMA-IR (4.7±0.7 vs 2.4±0.2 vs 1.8±0.2, p<0.0001) and Hep-IR (166±23 vs 96±6 vs 52±6, p<0.001 **Table 1**).

When analyzed as a whole group, and compared to CT, subjects with NAFLD had higher plasma levels of isoleucine and valine (branched-chain AA), tyrosine (aromatic AA), alanine, lysine, and glutamate, while plasma concentration of glycine was significantly decreased (all p<0.03, **Table 2**). However, most amino acids were increased only in NAFLD-Ob. Only alanine, glutamate, and among the branched chain only isoleucine and valine but not leucine concentrations were increased in NAFLD-NO compared to CT (**Table 2**). BCAA and AAA correlated also with liver enzymes, ALT (R=0.48, p<0.0001; R=0.37, p<0.004), AST (R=0.25, p=0.04; R=0.22 p=0.07), GGT (R=0.42, p=0.0008; R=0.31 p=0.01) (data not shown).

The GSG-index [glutamate÷(serine+glycine)]

Alterations of glutamate, glycine and serine often occurs in metabolic diseases (**Table 3**) and were confirmed also in this study. We first tested if the concentrations of these single amino acids and/or the ratio glutamate/(serine+glycine) (that we called GSG-index from the initials of the AA, see methods for details) were altered in NAFLD. We found that glutamate concentrations were significantly higher and glycine concentrations lower in patients with NAFLD compared to CT (**Table 2**). The GSG-index was significantly higher in NAFLD-NO compared to CT and even more in NAFLD-Ob (p<0.002), but no correlation was found with BMI. On the other hand the GSG-index was tightly correlated with increased liver enzymes, as a result of increased transamination, in particular with GGT (R=0.70. p<0.0001), but also with ALT and AST (R=0.42 and 0.40, p<0.002). (**Figure 2**). This is explained by the fact that GGT, but also ALT and AST, promote glutamate release during the transamination reactions (**Figure 1**). The GSG-index correlated well also with BCAA, in particular leucine, indicating an increased action of glutamate dehydrogenase (**Figure 2**). Glutamic acid alone was also associated, in a weaker way, with LFTs, while no association was found between LFTs and the concentration of the other amino acids that compose the index, i.e., glycine or serine.

Amino acids and insulin resistance

Only few amino acids had a significant correlation with Hep-IR. Glycine and histidine had a significant inverse correlation with Hep-IR (Spearman's rank coefficient R=-0.37, p=0.008 and

R=-0.29, p<0.05 respectively), while positive correlations were found with glutamate (R=0.29, p<0.04) and tyrosine (R=0.41, p=0.004). Only tyrosine (R=0.39, p<0.002) correlated with HOMA-IR. Peripheral insulin sensitivity (Rd/I) correlated only with tyrosine (-0.39, p=0.006).

The GSG-index correlated with Hep-IR (R=0.35 p<0.01), mildly with HOMA-IR (R=0.26 p<0.04) but not with Rd/I. No correlation was found between BCAA and Hep-IR, Rd/I or HOMA-IR. AAA were correlated with both Hep-IR (R=0.34, p<0.02), HOMA-IR (R=0.33, p<0.008) and Rd/I (R=-0.30, p=0.03 but only because of the association with tyrosine).

By using multivariable analysis we evaluated which among BCAA, AAA and GSG index was independently associated with Hep-IR and HOMA-IR and found that both GSG index (partial R=0.33, p=0.021) and AAA (partial R=0.32, p=0.027) were independently associated to Hep-IR (total R= 0.47, p=0.008), while only AAA (partial R=0.33, p=0.008) were independently associated to HOMA-IR (total R= 0.44, p=0.006).

Amino acids as markers of hepatic inflammation and fibrosis

We first tested which amino acid concentrations were altered in presence of hepatic ballooning and inflammation. Compared to CT, NAFLD with hepatocellular ballooning grade-2 had increased plasma concentrations of BCAA, AAAs, glutamate, alanine and lysine, while the concentrations of glycine and threonine were decreased **(Figure 3).** Similar results were found for inflammation although the concentrations of alanine were increased also in subjects with inflammation grade-0 (**Figure 4**). Amino acid concentrations were also associated to increased liver fibrosis (**Figure 5**) but concentrations observed in NAFLD with fibrosis score 3 and 4 were not significantly different than in those with fibrosis score 0-2.

The amino acids used to calculate the GSG-index are in some way involved in the synthesis of GSH (glutamic acid and glycine directly, while serine indirectly since it is a precursor of glycine) and of lipids associated to lipotoxicity (i.e., ceramides) (**Figure 1**). We therefore tested if GSG index was associated with alterations in liver histology. Subject with NAFLD had higher GSG-index independently of presence of ballooning or inflammation in liver biopsy. GSG-index was significantly increased with the degree of liver fibrosis, in particular with severe fibrosis F3-4 and the new index was able to discriminated NAFLD with F0-2 from F3-4 (**Figure 5**, p<0.0004 vs CT and p<0.04 vs F0-2)**.**

We used logistic regression analysis to evaluate which among BCAA, AAA and GSG index was independently associated with ballooning, inflammation or liver fibrosis. Compared to CT, the GSG index was able to discriminate better than BCAA and AAA subjects with inflammation (OR=36, p=0.01, vs OR= 1.02 and 1.01 p=ns), ballooning grade 2 (OR=28.5, p=0.03, vs OR= 1.03 and 1.01 p=ns), and fibrosis 3-4 (OR=122, p=0.004, vs OR= 1.04 and 1.00 p=ns). These results were confirmed after accounting for the different BMI.

Discussion

NAFLD is a recognized risk factor for the development of type 2 diabetes (T2DM) and cardiovascular disease (CVD) and patients with T2DM have an increased risk to develop NAFLD (29, 30). Most of T2DM patients have NAFLD even in presence of normal liver enzymes (31). Moreover, there is high risk for these patients to develop cirrhosis and hepatocellular carcinoma. For this reason, it is important to identify circulating markers of NAFLD for an early discovery and treatment of subjects with this disease.

Impact of IR on plasma AA concentration

Plasma amino acid concentrations have been implicated in the pathogenesis of NAFLD and progression to NASH but their causal role has not been established. AA concentrations are often found altered in metabolic diseases, including, type 2 diabetes, NAFLD and obesity, since they are also associated to insulin resistance (4, 6, 9, 32). Several studies have found aromatic amino acids (i.e., tyrosine and phenylalanine) increased in liver disease (6, 33, 34). Phenylalanine is irreversibly converted to tyrosine mainly in the liver where tyrosine is further metabolized. Increased tyrosine concentrations are often detected in patients with NAFLD (6, 34) possibly because of impairment in hepatic metabolism of this amino acid.

In the past years the attention has been focused on circulating branched chain amino acids (BCAA) that might play a role in promoting peripheral and hepatic insulin resistance (8, 9) and promote the onset of T2D (21, 35). However, increased BCAA concentrations might reflect impaired muscle rather than liver catabolism since the BCAA are metabolized principally in muscle (10). In subjects with NAFLD impaired suppression of BCAA during hyperinsulinemic euglycemic clamp (HEC) was proportional to impaired muscle glucose uptake during the clamp and peripheral insulin resistance (12). Women with abdominal obesity, but not those with lower

obesity, showed impaired suppression of leucine during HEC (36). Thus, it is not clear if subjects with NAFLD have an altered hepatic metabolism of amino acids or if AA are increased just because of obesity and insulin resistance, given that the majority of NAFLD patients are also obese. Consequently, we explored if the increased plasma concentration of amino acids, in particular BCAA, was a characteristic feature of all subjects with NAFLD or if AA were altered in presence of obesity and IR. We quantified plasma amino acid concentrations in non-diabetic subjects with NAFLD comparing obese to non obese and evaluated the impact of IR. We found NAFLD subjects more insulin resistant than control subjects when considered as a whole group, but when divided according to presence of obesity the differences between NAFLD-NO (i.e., those with BMI<30 kg/m2) and CT were reduced. Compared to CT non-diabetic NAFLD showed increased fasting values of glutamate, alanine, tyrosine, lysine, valine and isoleucine, i.e. glucogenic AA. Only fewer amino acids were increased in NAFLD-NO vs CT, i.e., glutamate, alanine, valine and isoleucine. When we evaluated the association with IR, hepatic IR correlated with most amino acids including BCAA, AAA and GSG, while only tyrosine correlated with peripheral IR. We found glutamate and tyrosine increased, while glycine and histidine decreased proportionally to hepatic IR. This is in agreement with previous studies that have often found glutamate and tyrosine increased in insulin resistant state such as obesity, T2DM, prediabetes and/or NAFLD (21, 32, 37, 38) also related to increased oxidative stress (6, 9) as well as to liver disease (19, 34). On the other hand glycine (12, 19-21, 38) and serine were found decreased (12, 23). A recent study the analysis of splanchnic fluxes based on the measurements in the artery and the hepatic vein showed that subjects with NAFLD have increased fasting hepatic import/degradation of glycine, serine and alanine while there is net release of glutamate, fatty acids, triglycerides and glucose (19). This remained true also during insulin infusion. We therefore conclude that elevated AA concentrations observed in NAFLD are very likely to be related to the peripheral resistance to the anticatabolic effect of insulin, that often accompany the defects in glucose metabolism and results in increased muscle proteolysis during fasting state and might explain some of the recent data on increased sarcopenia in subjects with NAFLD (39, 40).

Amino Acids and liver histology

Another important question is if AA concentrations are related to parameters of liver histology, like inflammation, ballooning and/or fibrosis. NAFLD/NASH is characterized by hepatic inflammation not always associated with whole body inflammation, but only liver biopsy can identify subjects with liver inflammation since circulating markers are still lacking. Several amino acids, released from muscle or synthesized in the liver, were previously implicated in NAFLD and the development of liver damage, among these BCAA and AAA. Thus, we have hypothesized that alterations in plasma amino acid concentrations might help identifying NAFLD subjects with hepatic inflammation and/or liver damage (i.e., fibrosis). We have found several plasma amino acid concentrations increased with inflammation and ballooning (**Figure 3 and 4**). Regarding liver fibrosis, glutamate was the amino acid that was more strongly associated with the severity of fibrosis (**Figure 5**). Glutamate is involved in many reactions: it is the product of AA transamination, once converted to α-ketoglutate is entering the TCA cycle, but it is also a substrate for intracellular glutathione (GSH) synthesis and turnover (**Figure 1**). Once produced, GSH is exiting the cell where it can be transaminated back by GGT releasing glutamate into the circulation (**Figure 1**). However, a previous report showed also that all hepatic enzymes linked to glutamate metabolism, except the cytosolic branched chain amino-acid transaminase 1 (BCAT1), were downregulated in the liver of patients with NASH and associated with increased circulating levels of glutamate (23). This was also confirmed in another study (32). We also have found that glutamate concentrations and GSG index increased in NAFLD and were strongly associated to GGT concentrations (**Figure 2**). Thus glutamate might reflect alteration in hepatic function possibly due to NASH, i.e. presence of inflammation, ballooning, oxidative stress and/or related to increased demand of glutathione. Other amino acids that participate to the synthesis of GSH have been previously found altered in NAFLD.

Previous studies showed that metabolic dysregulation is often associated to a reduction in glycine and serine concentration and an increase in the levels of valine and leucine (21, 23, 38). In this cohort of non-diabetic subjects with NAFLD we also found reduced concentrations of glycine and serine (a precursor of cysteine) and increased concentrations of glutamate, the last explained by the fact that as GSH is transaminated by GGT, glutamic acid becomes newly available, thus increasing its plasma concentration (**Figure 1**). Glycine plays an important role also in metabolic regulation, DNA methylation, intracellular redox balance, bile acid conjugation

and heme synthesis (22). Recently, glycine was shown to be the limited step for GSH synthesis (20). Our hypothesis was that in NAFLD there is an increased use of Serine and Glycine that determines the lower plasma concentrations, while Glutamate is increased due to increased transamination (**Figure 1**). We thus tested if the GSG-index (given by the ratio of glutamate/(serine+glycine)) was increased with the severity of liver histology and in particular liver fibrosis.

The GSG-index

A second result of our study was the development of a new metabolomics index, based on plasma concentrations of glutamate/(glycine + serine) that we called "GSG-index" from the initials of the amino acids. The GSG-index is composed of amino acids that participate to the synthesis of GSH (**Figure 1**) that is supposed to be increased in NAFLD/NASH (6, 20). GSH is an antioxidant molecule produced by several tissues in response to oxidative stress and increased production of reactive oxygen species (ROS) (41, 42). Oxidative stress is one of the mechanisms responsible of liver damage and is characterized by mitochondrial dysfunction, impaired oxidation, and production of ROS (43). This process increases the demand of GSH synthesis to counteract production of ROS by increasing intracellular GSH turnover (17, 44, 45). Amino acids like glycine, serine and glutamate are used for the synthesis of GSH (**Figure 1**). However, glutamate concentrations are often found increased in metabolic diseases (**Table 3**). This is explained by the fact that GGT promote glutamate release during the transamination of GSH (**Figure 1**).

We found the GSG-index correlated with Hep-IR and increased more in obese than non obese NAFLD (although it was not significantly associated to BMI or degree of liver steatosis). The GSG-index correlated well also with BCAA, in particular leucine, indicating a possible altered action of glutamate dehydrogenase that is responsible of the conversion of leucine and alanine to glutamate (**Figure 2**). Recent studies have confirmed the alteration of these 3 amino acids in NAFLD (19, 23). Preliminary data from our group indicate that the GSG-index is dynamic, since chronic treatment with pioglitazone, a PPAR-γ agonist known to improve IR, liver function and histology (46), was able to reduce the GSG-index together with the reduction in liver enzymes (47). Briefly in the ACT NOW study, where 441 IGT subjects were randomized to placebo plus diet or pioglitazone plus diet for 2.4 years, the GSG index was markedly reduced by pioglitazone proportionally to the reduction in IR in muscle, liver and adipose tissue and in liver enzymes.

This was evident especially in subjects that reversed to NGT, while in subjects that became diabetics there was an increase or no change in GSG-index (47).

Strengths and limits

One strength of our paper relies in the quantification amino acid concentrations (by GCMS using labeled internal standards) in non-obese non-diabetic subjects with biopsy proven NAFLD, thus avoiding the confounding effect of obesity, insulin resistance and hyperglycemia. Moreover, we have provided direct measurement of hepatic-IR, quantified using the stable isotope tracer technique. We provided evidences that alterations in concentrations depends on obesity and IR status, since only few amino acids (valine, isoleucine, alanine and glutamate) were significantly elevated in NAFLD-NO compared to CT. We have developed a new index based on the ratio of glutamate/(serine+glycine) that was independently associated to severe liver damage and able to discriminate fibrosis F0-2 from F3-4 independently of BMI. Moreover, the GSG-index has the advantage (as a ratio) to be calculated also in qualitative and semi-quantitative studies, where appropriate labeled internal standards are not always used, and to be used as a metabolomics biomarker in future studies.

Limits: this study is cross-sectional and the GSG-index needs to be evaluated in a prospective study. However, preliminary data from our group have shown that 2.4 years of treatment with pioglitazone decreases the GSG-index proportionally to the improvement in insulin sensitivity (in muscle, liver and adipose tissue) and to the decrease in liver enzymes (47).

In conclusion, increased plasma amino acid concentrations are observed more in obese than in non-obese NAFLD, and are strongly associated to increased insulin resistance. Subjects with more advanced liver damage display increased values of BCAA (isoleucine, valine), AAA (tyrosine) and GSG-index (glutamate) but only the new GSG-index was able to discriminate mild to severe liver fibrosis becoming a possible biomarker of severity of liver disease.

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Table 1 clinical characteristic of study subjects

	Controls n=20	Non obese NAFLD n=29	Obese NAFLD n=15	P value CT vs NAFLD	P value Non obese NAFLD vs CT	P value Obese NAFLD vs CT
Gender (M/F)	7/13	24/5	12/3			
Inflammation (y/n)	-	4/25	1/14			
Balloning (0/1/2)	-	3/12/14	2/4/9			
Fibrosis (0,1-2,3-4)	-	15/9/5	1/6/8			
Age (years)	41±3.4	43±2.1	38.1±2.8	ns	ns	ns
BMI (kg/m²)	25±0.9	26±0.5	32±2.2	0.001	ns	<0.0001
Waist (cm)	84±3.5	90±6.7	104±2.3	0.0007	0.03	<0.0001
ALT (U/L)	12.3±1.3	62.8±5.6	96.3±9.7*	<0.0001	<0.0001	<0.0001
AST (U/L)	19.0±1.4	41.6±7.2	48.9±3.5*	<0.0001	<0.0001	<0.0001
GGT (U/L)	18.5±3.4	99.9±17.1	106.4±38.3	<0.0001	<0.0001	<0.0001
Glucose (mg/dL)	91.3±2.3	94.9±1.7	100.5±3.7	ns	ns	0.02
Insulin (mU/L)	7.8±0.8	10.2±0.6	18.8±2.3*	0.003	ns	<0.0001
Cholesterol (mg/dL)	210.0±10.5	189.3±5.7	200.9±10	ns	ns	ns
HDL (mg/dL)	51.1±3.0	46.1±1.9	44.5±3.9	ns	ns	ns
LDL (mg/dl)	134.4±8.3	122.6±5.7	137.0±7.6	ns	ns	ns
TG (mg/dL)	83.8±7.9	97.5±8.1	114.3±16.2	ns	ns	0.05
HOMA-IR (mmol/l x mU/l)	1.81±0.23	2.41±0.17	4.74±0.66*	0.005	0.02	<0.0001
Hepatic-IR (umol/min kg x mU/l)	52±6	96±6	166±23*	0.0003	0.0012	<0.0001
Peripheral IR (Rd/I) (umol/min kg x (mU/l)$^{-1}$)	1.65±0.24	1.08±0.11	0.55±0.06*	0.0002	0.0017	<0.0001

*p<0.05 NAFLD-Ob vs NAFLD-NO

Table 2 Plasma amino acid concentrations

Amino Acid (mmol/l)	Controls n=20	Non obese NAFLD n=29	Obese NAFLD n=15	P value CT vs NAFLD	P value Non obese NAFLD vs CT	P value Obese NAFLD vs CT
Alanine#	328.1±20.4	374.3±13.6	396.5±20	0.015	0.05	0.01
Valine#	117.0±4.7	141.3±4.7	151.7±8.5	0.0002	0.002	0.0002
Isoleucine#§	56.2±3.6	67.2±2.1	69.8±5.1	0.005	0.015	0.01
Leucine§	139.9±6.2	146.8±3.8	168.1±11.1*	0.08	ns	0.006
Tyrosine#§	66.1±3.7	75.3±2.8	94.6±6.6*	0.005	0.09	<0.0001
Phenylalanine#§	54.3±1.8	53.9±1.6	62.6±2.7*	ns	ns	0.004
Lysine§	140.5±4.7	153.4±4.6	161.9±7	0.02	0.07	0.01
Threonine#§	73.4±4.6	66±2	65.2±4.4	ns	ns	ns
Proline#	171.8±16.0	207.6±15.3	224.7±35.3	ns	ns	ns
Hystidine#	96.4±3.9	90.3±3.7	109.4±10.3*	ns	ns	ns
Glutamate#	119.0±18.4	196.2±16.7	218.8±31.4	0.001	0.007	0.003
Serine#	105.2±3.3	97.5±3.12	108.2±4.7*	ns	ns	ns
Glycine#	205.9±9.7	187.8±5.8	179.2±7.6	0.03	ns	0.03
GSG-index	0.38±0.05	0.69±0.07	0.75±0.09	0.0004	0.002	0.002

*p<0.05 NAFLD-Ob vs NAFLD-NO; # glucogenic amino acids, § ketogenic amino acids, BCAA: leucine, isoleucine and valine; AAA: Tyrosine and phenylalanine.

Table 3. Articles that show alterations in AA metabolism in obesity/NAFLD

Reference	Study Population NAFLD and/or T2DM	Key results
Bianchi et al.,(2003)	39 biopsy-proven NAFLD and 10 control subjects	Reduced serine and glycine in NAFLD. Greater suppression of BCAA and serine during insulin clamp, associated with higher M value
Newgard et al., (2009)	74 obese and 67 lean subjects with insulin resistance	increased BCAA, tyrosine, phenylalanine, alanine, glutamate/glutamine and decreased glycine in obese subjects
Kalhan et al., (2011)	Non diabetic subjects with hepatic steatosis (n = 11) or NASH (n = 24) compared to healthy controls (n = 25).	higher levels of BCAA, phenylalanine, tyrosine and glutamate, but lower plasma GSH and GSH metabolites in NASH
Wang-Sattler et al., (2012)	Individuals who developed insulin resistance or T2D over 7 years	Decreased glycine concentration is predictors of impaired glucose tolerance and of T2D
Cheng et al., (2012)	Two cohorts free of diabetes Framingham Heart Study (FHS; n=1015); the Malmo¨ Diet and Cancer Study (MDC; n=746) with a 12-year follow-up	Plasma glutamate concentration was strongly associated with insulin resistance and circulating TG. Low glutamine and high glutamate concentrations were associated to incidence of diabetes in FHS
Wurtz et al, (2012)	1,873 Finnish individuals followed up for 6.5 years	Increased BCAA, phenylalanine, tyrosine and reduced glycine were all associated with glycemia and insulin sensitivity at baseline and predictors of fasting and/or postchallenge glucose in this study
Floegel et al (2013)	Two cohorts with a 7-year follow-up and diabetes relevant traits	higher plasma concentrations of phenylalanine, isoleucine, tyrosine, and valine were associated with increased risk of T2D and glycine to be associated with reduced risk of T2D.
Lynch et al (2014)	Review on current knowledge on the metabolic effects of BCAA	The authors conclude that "increased BCAA levels are more likely to be a marker of loss of insulin action and not, themselves, causative"
Mardinoglu et al. (2014)	genome-scale metabolic model of liver in patients with NAFLD.	Nonessential amino acids serine, glycine, glutamate, glutamine, aspartate, asparagine and alanine, and essential amino acids valine and methionine are involved in the appearance of NASH. NASH patients have severe serine deficiency
Lake et al. (2015)	frozen liver from 17 healthy, 4 steatosis, 14 NASH fatty, and 23 NASH not fatty	Increased BCAA and tyrosine, and increased BCAT1 activity in liver of patients with NASH.
Palmer al. (2015)	96 subjects from the Insulin Resistance Atherosclerosis Study at the extremes of the Insulin sensitivity distribution	Decreased glycine and increased valine, leucine, and combined glutamine and glutamate was associated to conversion to T2D (76 converters; 70 nonconverters)
Kawanaka et al., 2015)	137 patients with NAFLD and liver biopsy	Plasma BCAA levels, and BCAA-to-Tyr ratio values were negatively associated with the fibrosis stage and Tyr levels increased with increasing fibrotic staging. Tyr levels were also correlated with HOMA-IR.
Hyotylainen, et al. (2016)	measurements in the artery and the hepatic vein of 8 NAFLD and 8 healthy subjects.	increased fasting hepatic import/degradation of glycine, serine, tyrosine, cysteine and alanine while there is net release of glutamate, fatty acids, triglycerides and glucose in NAFLD.
Mardinoglu et al. (2017)	86 subjects with varying degrees of hepatic steatosis (HS)	Fasting plasma levels of glycine and serine showed significantly negative correlations with HS. Glycine and serine are the limiting substrates for the de novo synthesis of GSH

Figure legend

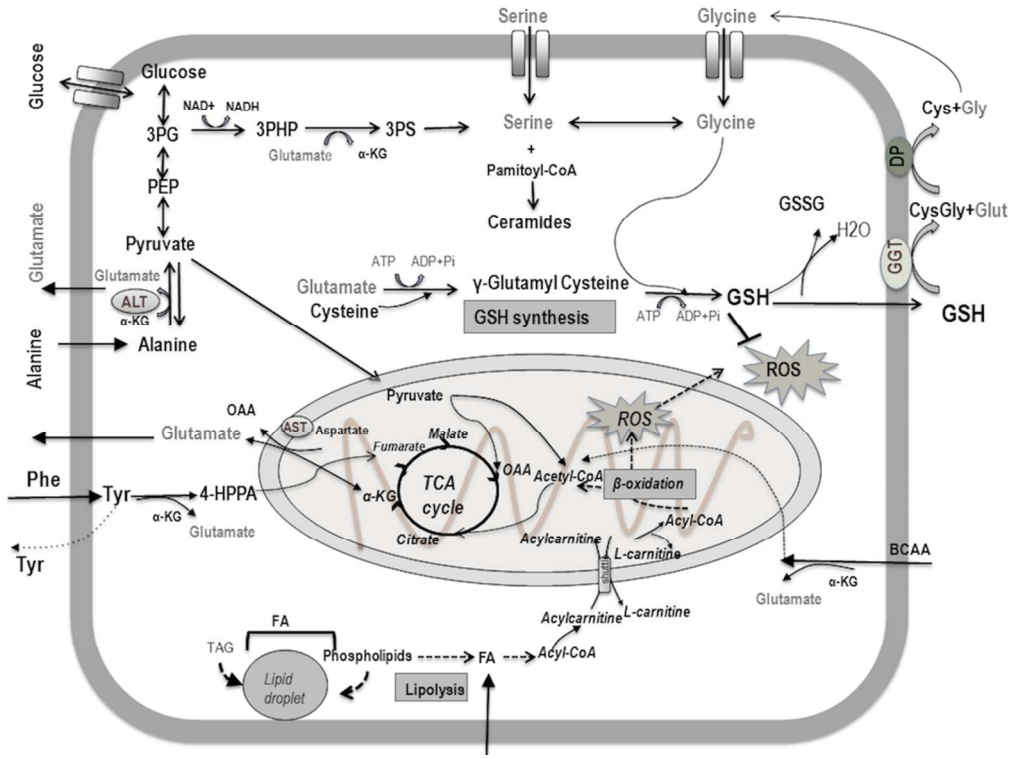
FIGURE 1 the figure shows the hepatic metabolic pathway in which amino acids (in particular the components of the GSG index glutamate, serine and glycine) are involved. In presence of NASH, inflammation and oxidative stress are increased. Reactive oxygen species (ROS) stimulate the synthesis of glutathione (GSH) from glutamate, glycine and cysteine. Once GSH reaches the cell surface it is transaminated by GGT and glutamate is released into the bloodstream. Glutamate is also produced from transamination of alanine and aspartate. Abbreviations: Phosphoenol-pyruvate (**PEP**), 3-phosphoglycerate (**3PG**), 3-3-phosphohydroxypyruvate **(3PHP),** 3-phosphoserine **(3PS),** cysteine (**Cys**), glycine (**Gly**), glutamate (**Glut**), α-ketoglutarate (**α-KG**), oxaloacetate (**OAA**), Glutathione (**GSH**), Glutathione disulfide (**GSSG**), Dispeptidase (**DP**), Gamma Glutamyl Transferase (**GGT**), Alanine Amino Transferase (**ALT**), Fatty Acid (**FA**), Triacylglycerols (**TAG**), Phenylalanine (**Phe**), Tyrosine (**Tyr**), 4-Hydroxyphenylpyruvic acid (**4-HPPA**), Branched chain amino acids (**BCAA**).

FIGURE 2: GSG-index was strongly correlated with circulating levels of BCAA, and liver enzymes, in particular GGT

FIGURE 3: top panel shows fold changes (compared to non-NAFL) in amino acid concentration in subjects with NAFLD/NASH with different degrees of hepatic ballooning at liver biopsy (scores 0, 1 and 2; n=5, 16 and 23 respectively). Bottom panels show how GSG-index, BCAA and AAA are increased in NAFLD with liver ballooning compared to non-NAFLD (CT).

FIGURE 4: top panel shows fold changes (compared to non-NAFL) in amino acid concentration in subjects with NAFLD/NASH, in presence (n=39) or not (n=5) of hepatic inflammation at liver biopsy. Bottom panels show how GSG-index, BCAA and AAA are increased in NAFLD with liver inflammation compared to non-NAFLD (CT) or NAFLD with no inflammation at liver biopsy.

FIGURE 5: top panel shows heat map of fold changes (compared to non-NAFL) in amino acid concentrations in subjects with NAFLD/NASH according to degree fibrosis at liver biopsy. Bottom panels show how GSG-index was able to better discriminate NAFLD F0-2 (n=32) vs F3-4 (n=12) vs non-NAFLD compared to BCAA and AAA.



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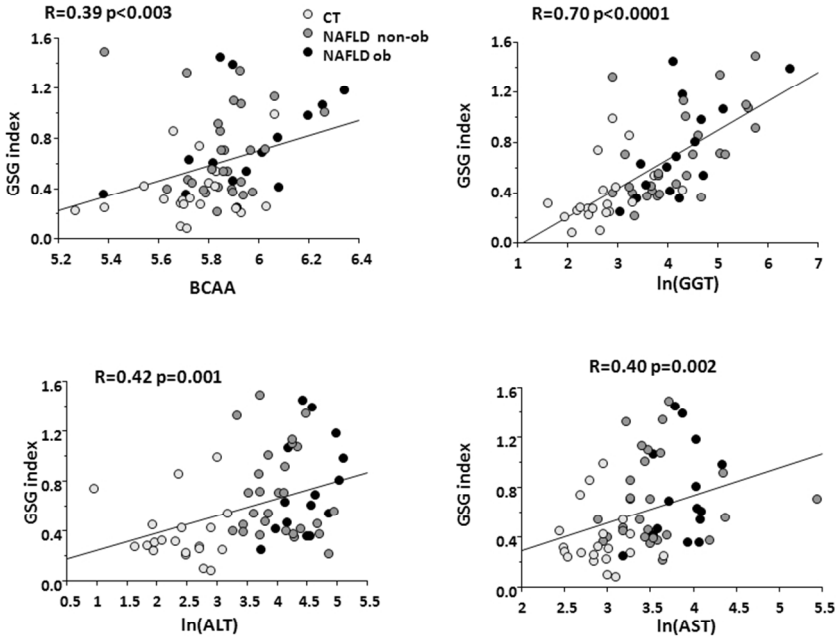

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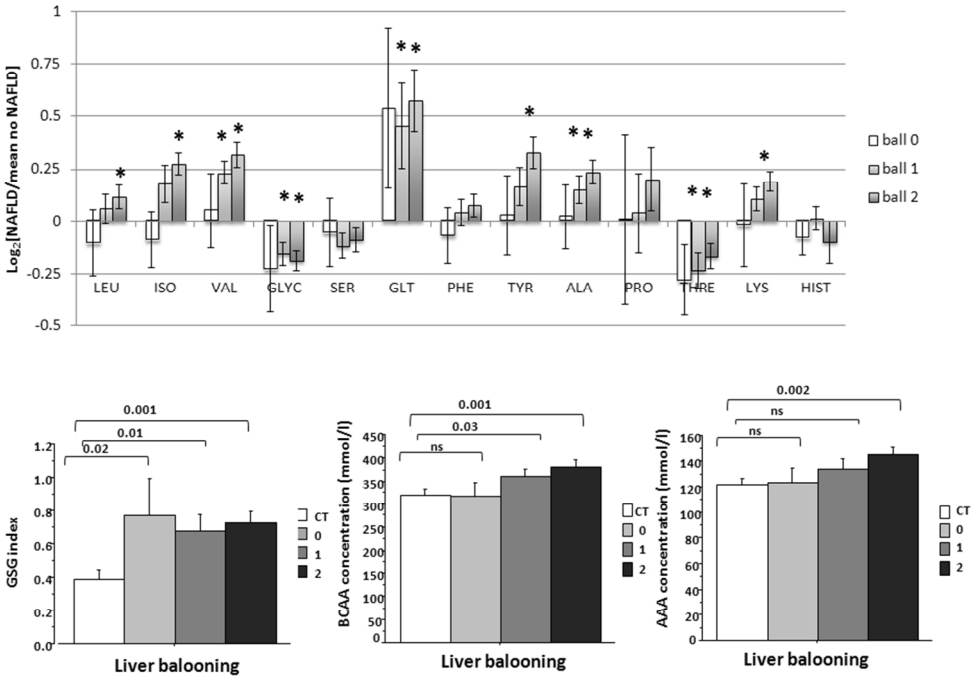

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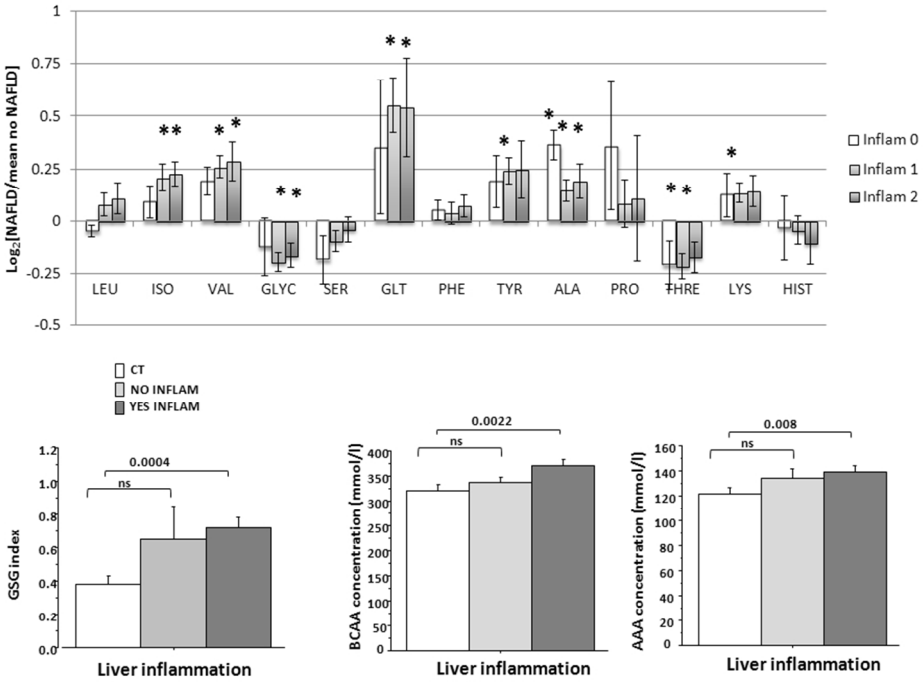

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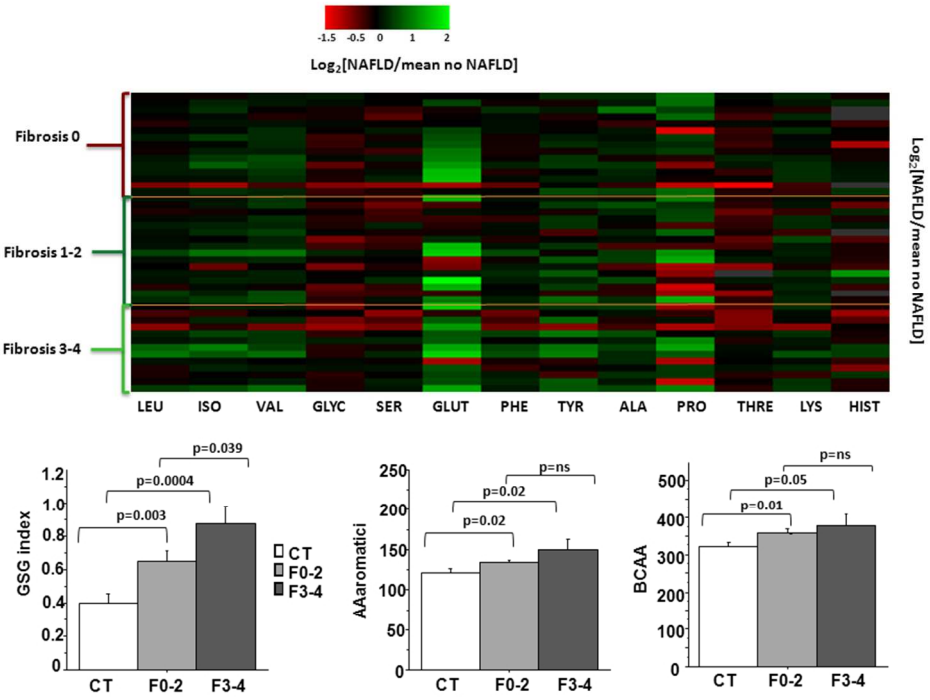

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