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Independent association between low serum amylase and non-alcoholic fatty liver disease in asymptomatic adults: A cross-sectional observational study

Short title: Low serum amylase and non-alcoholic fatty liver disease

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Key words: serum amylase, NAFLD, metabolic syndrome, diabetes

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ABSTRACT

Objectives: Low serum amylase (LSA) was reported to be associated with obesity, metabolic syndrome (MetS), and diabetes. However, it is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of MetS and insulin resistance. Therefore, we performed a clinical epidemiological study to investigate this potential association.

Design: A cross-sectional observational study with multivariate analysis.

Setting: Subjects were recruited in a healthcare centre in Saitama, an eastern district of Japan, near Tokyo.

Participants: A total of 1,475 asymptomatic adults aged 30–79 years who underwent detailed medical check-ups and who regularly consumed small amounts of alcohol (< 20 g/day).

Outcome measures: Serum amylase, cardiometabolic risk factors, NAFLD determined by ultrasound, MetS determined by Adult Treatment Panel-III criteria, and diabetes were assessed.

Results: The prevalence of NAFLD increased significantly from 22.5% to 42.4% (all grades) and from 9.2% to 24.0% (moderate or severe grade) from the highest to the lowest quartile of serum amylase. Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase, the lowest quartile of serum amylase was significantly associated with any-grade NAFLD and with moderate to

severe NAFLD, even after adjusting for MetS or diabetes. The association between LSA and any-grade NAFLD disappeared after further adjustment for body mass index or waist circumference, whereas the association between LSA and moderate or severe NAFLD remained statistically significant [odds ratios (95% confidence interval), 2.01 (1.07–3.78) and 2.06 (1.09–3.87), respectively, both $P = 0.01$].

Conclusions: Our results suggest that LSA may be a valuable marker for moderate or severe NAFLD in asymptomatic adults independent of MetS, diabetes, and obesity. These results warrant confirmation in further studies.

(275/300 words)

ARTICLE SUMMARY

Article focus

• The clinical relevance of low serum amylase (LSA) is unclear, but it is putatively associated with obesity-related metabolic abnormalities.

• It is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of cardiometabolic disease and insulin resistance.

Key messages

• Our results suggest that LSA is associated with NAFLD independent of metabolic syndrome, diabetes, and obesity.

• LSA may be an independent marker for moderate or severe NAFLD.

Strengths and limitations of this study

• A possible association between LSA and NAFLD was evaluated after fully adjusting for relevant confounding factors.

• NAFLD was diagnosed with ultrasound in this study. Other methods, such as computed tomography and magnetic resonance imaging, may provide more precise assessment of NAFLD.

• This was an observational study and the cause–effect relationship is unknown.

INTRODUCTION

Abnormal serum amylase levels generally reflect overall dysfunction of the pancreas or salivary glands. Although the clinical relevance of elevated serum amylase levels has been extensively studied in relation to various conditions, including acute pancreatitis, pancreatic cancer, ectopic amylase-producing tumours, abdominal trauma, and kidney dysfunction [1-5], the clinical relevance of low serum amylase (LSA) has not been examined. LSA is conventionally considered as a crude marker for diffuse pancreas destruction secondary to pancreatic diseases, such as advanced chronic pancreatitis and cystic fibrosis [6,7]. LSA or low pancreatic amylase is also associated with insulin deficiency in patients with type 1 diabetes and in patients with longstanding type 2 diabetes [8-11]. In our recent community-based study, LSA, defined as a serum amylase concentration of < 60 IU/l, was observed in 25% of asymptomatic individuals [12]. LSA is also associated with metabolic syndrome (MetS), a cluster of cardiovascular risk factors, and diabetes [12,13]. However, obesity is thought to be the strongest predictor of LSA in the asymptomatic general population [12].

In the last two decades, there has been a marked increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide, along with an obesity pandemic [14,15]. NAFLD often progresses to a more severe condition, non-alcoholic steatohepatitis (NASH), and increased hepatic fibrosis is a significant histological feature of advanced NASH. NAFLD is considered to be a hepatic manifestation of

MetS and insulin resistance [14-18], which suggests that NAFLD consists of a wide spectrum of cardiometabolic diseases. It is also possible that NAFLD may reflect more pronounced insulin resistance compared with MetS in certain clinical settings, including in individuals without diabetes or obesity [17,19,20]. In this context, we hypothesised that LSA may be associated with NAFLD independently of MetS and type 2 diabetes. The aim of this study was to test this hypothesis and to investigate the clinical relevance of LSA, which is often observed in clinical practice. Therefore, we examined the associations among serum amylase, cardiometabolic risk factors, NAFLD, hepatic fibrosis, MetS, and diabetes in a cross-sectional study of asymptomatic adults.

METHODS

Protocol and subjects

The present report represents a series of observational studies performed in collaboration with Josai University, Sakado, Japan, and Social Insurance Omiya General Hospital that have been conducted to elucidate the relationships between lifestyle-related diseases and cardiometabolic risk factors. We recruited, at random, asymptomatic subjects aged 30–79 years who underwent thorough annual medical check-ups at the Social Insurance Omiya General Hospital, Saitama, Japan, between April 2009 and March 2010. The protocol was approved by The Ethics Committee of Josai University and the Council of the Hospital, and informed consent was obtained

from all participants.

Subjects with C-reactive protein ≥ 10.0 mg/l, estimated glomerular filtration rate (eGFR) ≤ 35 ml/min/1.73 m^2, serum amylase ≤ 30 IU/l or ≥ 200 IU/l, and those suspected of having cancer were excluded from the study. Subjects who habitually consumed alcohol ≥ 20 g per day were also excluded from the study. Subjects positive for hepatic B virus surface antigen were also excluded. Hepatic C virus infection was not measured in this study. We also excluded subjects with other active liver diseases not examined, by excluded those with elevated serum liver enzyme levels (approximately three times the upper limit of normal): alanine aminotransferase (ALT) ≥ 150 IU/ml, aspartate aminotransferase (AST) ≥ 150 IU/ml, or γ-glutamyltransferase (GGT) ≥ 150 IU/ml. Consequently, a total of 1,475 individuals were included in this study.

Laboratory measurements

Laboratory and anthropometric tests, and an abdominal ultrasound were performed in the early morning after an overnight fast. Serum parameters were measured using an AutoAnalyzer (Hitachi, Tokyo, Japan). The serum amylase level was measured using an enzymatic method (L-type Amylase; Wako, Tokyo, Japan) with a detection limit of 1.7 IU/l, and a run-to-run coefficient of variation of < 5.0%.

Abdominal ultrasound for the detection of fatty liver was carried out by

echography specialists at the Social Insurance Omiya General Hospital. Fatty liver in the eligible subjects was defined as NAFLD because they consumed < 20 g of alcohol per day [14]. The severity of NAFLD was graded into three categories: mild NAFLD, a slight increase in liver echogenicity with normal visualisation of the diaphragm and the portal veins; moderate NAFLD, a moderate increase in liver echogenicity with slightly impaired visualisation of the diaphragm and the portal veins; and severe NAFLD, a substantial increase in liver echogenicity with poor or no visualisation of the diaphragm and the portal veins. Since it is possible that mild NAFLD could include a normal liver, we defined overt NAFLD as moderate or severe NAFLD. The following liver fibrosis scores, the AST/ALT ratio [21], FIB-4, and AST to platelet ratio index (APRI), were calculated in all patients, using previously published formulae:

FIB-4 = age (years) × AST/platelet count (10^9/l) × $ALT^{1/2}$) [22];

APRI = [(AST/the upper limit of normal of AST = 35) × 100]/platelet count (10^9/l) [23].

The diagnosis of MetS was based on the Adult Treatment Panel-III criteria [24] with the following cut-off limits: 1) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; 2) triglyceride ≥ 150 mg/dl; 3) high-density lipoprotein–cholesterol < 40 mg/dl for men and < 50 mg/dl for women; 4) fasting plasma glucose ≥ 100 mg/dl; and 5) waist circumference ≥ 90 cm for men and ≥ 80 cm for women. Subjects meeting three or more of these criteria, including treatment for any of these disorders, were defined as having MetS. Diabetes was defined as fasting plasma

glucose ≥ 126 mg/dl or glycosylated haemoglobin (HbA1c) ≥ 6.5% (by the National Glycoprotein Standardization Program (NGSP)) according to the American Diabetes Association criteria [25], or treatment with oral hypoglycaemic drugs or insulin. HbA1c (Japan Diabetes Society [JDS]) was converted to HbA1c (NGSP) units using the officially certified formula: HbA1c (NGSP) (%) = 1.02 × JDS (%) + 0.25% [26].

Since serum amylase can be affected by kidney function because of its excretion by the kidney [4,5], eGFR was considered as a confounding factor and was calculated using the following equation for Japanese subjects [27]: eGFR (ml/min/1.73 m^2) = 194 × serum $Cr^{-1.094}$ × $age^{-0.287}$ (if female) × 0.739. Here, Cr = serum creatinine concentration (mg/dl).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or median (interquartile range). Subjects were divided into quartiles according to serum amylase levels, where Q1 is the highest quartile and Q4 is the lowest quartile. *P*-values for continuous variables were determined using analysis of variance (ANOVA) or the Mann-Whitney *U* test, and for categorical variables using the χ^2 test. Highly skewed values (ALT, AST, GGT, APRI, and triglyceride) were log-transformed before analysis. Subjects were also stratified into four groups according to the grade of NAFLD (i.e., normal, mild, moderate, and severe NAFLD). Multivariate logistic regression models were used to

examine the associations of quartiles (Q2–4) of serum amylase with any-grade NAFLD (NAFLD-AG), and with overt NAFLD relative to Q1, controlling for relevant confounding factors, including MetS, diabetes, and body mass index (BMI) or waist circumference (WC). To elucidate any association between LSA and NAFLD, we examined the associations between LSA and NAFLD-AG, and between LSA and overt NAFLD. This analysis yielded odds ratios (OR) and 95% confidential intervals (95% CI). Tests for linear trends (*P* for trend) were calculated by treating the quartiles as a continuous variable (i.e., 1–4), and the same model analysis was conducted. Statistical analyses were performed using SPSS software version 18.0 (SPSS-IBM, Chicago, IL, USA) and Statview version 5.0 (SAS Institute, Cary, NC, USA). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The clinical characteristics of the subjects according to the quartile of serum amylase are shown in Table 1. The mean and median values of most clinical parameters, including platelet count and eGFR, increased significantly with as serum amylase decreased, although there was a significant trend for subjects with lower amylase levels to be younger. No significant trend in HbA1c was observed against quartiles of serum amylase. The prevalence of NAFLD-AG and overt NAFLD increased significantly, from 22.5% to 42.4% and from 9.2% to 24%, respectively, from the highest (Q1) to the

lowest serum amylase quartile (Q4). Log-transformed serum ALT and GGT increased significantly, whereas the AST/ALT ratio, APRI, and FIB-4 decreased significantly with decreasing serum amylase quartile.

When subjects were stratified according to NAFLD grade, serum amylase decreased significantly with advancing grade of NAFLD (Figure 1). When subjects were further stratified according to sex, there was no significant difference between men and women. However, when the subjects were stratified according to high or low BMI (Figure 2), serum amylase levels were significantly lower in overweight/obese subjects (BMI ≥ 25.0 kg/m^2, mean ± SD BMI, 27.3 ± 2.3 kg/m^2, n = 1,121) than in lean subjects (BMI < 25.0 kg/m^2, mean ± SD BMI, 21.6 ± 2.0 kg/m^2, n = 354). Serum amylase levels in lean subjects decreased significantly with advancing grade of NAFLD, whereas those in obese subjects did not. Significant differences between lean and obese subjects were only observed only in subjects with a normal liver or mild NAFLD. We did not conduct an analysis by further stratification according to MetS or diabetes because of the small proportion of subjects (both, < 10%).

Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase (≥ 90 IU/l, Q1), the lowest quartile (< 60 IU/l, Q4) was significantly associated with NAFLD-AG even after adjusting for confounders plus MetS or diabetes (Table 2, Models 4 and 5). In these conditions, MetS (OR: 3.66, 95% CI: 2.37–5.65) and diabetes (OR: 1.96, 95% CI: 1.25–3.05) were significantly

associated with Q4 of serum amylase (data not shown). However, these associations were markedly attenuated and were no longer statistically significant after adjusting for clinical confounders plus BMI or WC (Models 6 and 7), although there was a trend towards an association among the quartiles of serum amylase.

When we excluded subjects with mild grade NAFLD (n = 242) and repeated the analysis (Table 3), we detected stronger significant associations between Q4 and overt NAFLD. These associations were also moderately attenuated after additional adjustment for BMI or WC, but they remained statistically significant (Models 6 and 7). In this condition, BMI (OR: 1.65, 95% CI: 1.51–1.80) and WC (OR: 1.21, 95% CI: 1.17–1.25) were significantly associated with overt NAFLD (data not shown).

DISCUSSION

For the last few decades, NAFLD has been considered as a manifestation of MetS and insulin resistance [14-18]. In this context, the present study showed that LSA was significantly associated with NAFLD-AG in asymptomatic adults, independent of MetS, diabetes, and sex. Furthermore, LSA may be indicative of overt NAFLD, i.e., moderate or severe NAFLD, independent of obesity. These findings are not inconsistent because NAFLD may reflect insulin resistance, more so than MetS, in individuals without diabetes or obesity [17,19,20].

This study provided evidence that NAFLD can occur in lean individuals, who

accounted for 37.8% (62/104) of subjects with moderate NAFLD and 19.3% (11/57) of subjects with severe NAFLD (Figure 2). This is consistent with the results of multivariate logistic analysis, where there was a significant trend for NAFLD against quartiles of serum amylase, and the significant association between the lowest quartile of serum amylase and NAFLD remained after adjusting for BMI and WC (Tables 2 and 3). Notably, we found no significant difference in serum amylase between lean and obese subjects with overt NAFLD, suggesting that the effects of obesity on serum amylase are apparent in subjects with a normal liver or with mild NAFLD, but not in subjects with overt NAFLD. Several studies have shown that NAFLD occurs in non-obese people, although the prevalence of NAFLD in non-obese subjects is lower than that in obese subjects [19,20,28]. Therefore, other than obesity, factors not examined in this study might also explain the association between LSA and NAFLD. Insulin resistance and obesity-related hormones, such as leptin, may be potential factors, because insulin resistance and energy imbalance are common pathophysiological findings in obese individuals [17,29]. Indeed, obesity plus insulin resistance is more closely associated with metabolic abnormalities and NAFLD than is obesity without insulin resistance [17]. Hyperinsulinaemia caused by insulin resistance stimulates *de novo* lipogenesis in the liver through sterol regulatory element binding protein-1c (SREBP-1c) [30], irrespective of the influx of fatty acid from a meal and lipolysis in adipose tissues. Additionally, we recently reported a latent association between LSA and

insulin resistance in a small study of asymptomatic middle-aged adults [31], supporting the hypothesis that LSA may be associated with NAFLD, at least in part through insulin resistance. However, further large epidemiological studies evaluating insulin resistance are needed to confirm our hypothesis.

As another explanation, systemic ectopic fat deposition in organs including the pancreas (i.e., fatty pancreas) might contribute to the observed associations because intrapancreatic fat, particularly intralobular pancreatic fat, was reported to be associated with NAFLD and MetS [32,33]. An animal study showed that obese mice had a heavier pancreas and more intrapancreatic fat [34]. Therefore, systemic fat deposits in multiple organs may be a common cause underlying the association between NAFLD and overall pancreatic dysfunction, which may result in impaired exocrine function, characterised as LSA. Gene polymorphisms of patatin-like phospholipase domain containing 3 (PNPLA3) may also contribute to the strength of these associations. For example, a common variant of the PNPLA3 gene (rs738409) was reported to be associated with increased hepatic fat content (i.e., NAFLD) [35]. In the mouse liver, adiponutrin/PNPLA3 gene expression is under the direct transcriptional control of SREBP-1c, in response to insulin [36]

Similar to an earlier study [12], HbA1c was not associated with LSA, probably because most subjects in the lowest quartile of serum amylase in this study had no or only mild insulin resistance or mild hyperinsulinaemia, which is likely compensated for

and results in euglycaemia. In such conditions, fasting plasma glucose would increase linearly, as observed in Table 1, in response to insulin resistance, particularly in the early stage of diabetes [37,38].

To date, some markers have been considered for screening for NAFLD and mild hepatic fibrosis [39-41]. In this study, the AST/ALT ratio decreased significantly with the decreasing quartile of serum amylase. However, while the platelet count increased significantly, both APRI and Fib-4 decreased (Table 1). Consequently, hepatic fibrosis is unlikely to be associated with NAFLD in this study. A plausible explanation is that this study mostly consisted of non-obese individuals, with a small proportion having MetS and diabetes, resulting in a lower likelihood of advanced hepatic fibrosis and NASH. In addition, more than half of the subjects in this study had repeatedly undergone medical check-ups, which may promote consciousness of healthcare and favourable lifestyles.

Limitations

Several limitations should be mentioned. First, NAFLD was diagnosed by ultrasound rather than by histological examination, the gold standard technique for the diagnosis of NAFLD. Thus, NAFLD and the degree of hepatic fibrosis could be inaccurately evaluated, particularly in the early stages. Nevertheless, the ultrasound method is effective for screening NAFLD, with an overall sensitivity of 89% and specificity of 93% [42]. Despite this, in cases of mild NAFLD with a small amount of fat (< 30%),

the sensitivity is reduced. For this reason, we examined the association of LSA with NAFLD-AG and with overt NAFLD. Second, hepatitis C virus infection was not measured in this study. However, the prevalence of hepatitis C infection was reported to be 0.49–0.98% in Japanese blood donors [43,44]. Therefore, hepatitis C infection is unlikely to profoundly modify the association between LSA and NAFLD, although hepatitis C infection can contribute to the development of NAFLD [45]. Third, because of the cross-sectional nature of the study, we could not determine the cause–effect relationship between LSA and NAFLD. However, in a previous retrospective study [12], subjects with LSA at baseline were more likely to develop MetS-related metabolic abnormalities 5 years later. A longitudinal prospective study or a clinical intervention trial will be needed to elucidate the causality of the associations reported here.

Conclusion

Our results suggest that LSA may be associated with NAFLD, particularly moderate or severe NAFLD, in asymptomatic adults. This association was independent of MetS, diabetes, and obesity. Further studies are needed to confirm the observed associations, and to explore the clinical relevance of LSA.

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Contributors

KN, HM, and MK designed the collaborative project; KN, HO, TM, and HF collected and analysed the data; KN, HO, MS, and YH researched and evaluated the literature; and KN wrote the first draft of the manuscript. All authors reviewed and edited the manuscript, and approved the final version of the manuscript.

Data sharing statement There are no additional data available.

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Figure Legends

Figure 1 Serum amylase levels according to the grade of NAFLD and sex. Serum amylase levels decreased significantly with advancing grade of NAFLD in both sexes (ANOVA, both $P = 0.0001$). There was no significant difference between men and women (two-way ANOVA, $P = 0.45$). *Number of subjects in each group. Bars represent standard errors.

Figure 2 Serum amylase levels according to the grade of NAFLD and obesity. Obesity was defined as BMI ≥ 25.0 kg/m^2. Serum amylase levels decreased significantly with advancing grade of NAFLD in lean subjects (ANOVA, $P = 0.01$), but not in obese subjects ($P = 0.33$). An overall significant difference was observed between lean and obese subjects (two-way ANOVA, $P = 0.03$). *Number of subjects in each group; $^{\dagger}P = 0.0001$ and $^{\ddagger}P = 0.02$ for lean versus obese subjects (Mann–Whitney U test). Bars represent standard errors.

Table 1. Characteristics of subjects stratified according to quartiles of serum amylase

	Total	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* value
n	1,475	369	371	381	354	
Age, y	55.1 ± 12.2	58.4 ± 11.8	56.4 ± 12.2	52.9 ± 11.7	52.7 ± 12.0	< 0.0001
Men, n (%)	747 (50.6)	178 (48.2)	176 (47.4)	205 (53.8)	188 (53.1)	0.19
BMI, kg/m^2	23.0 ± 3.2	21.9 ± 2.7	22.6 ± 3.2	23.3 ± 3.1	24.1 ± 3.4	< 0.0001
WC, cm	80.8 ± 8.9	78.2 ± 8.3	79.9 ± 8.8	81.8 ± 8.8	83.2 ± 9.1	< 0.0001
Systolic blood pressure, mmHg	120 ± 18.9	119 ± 18.5	119 ± 18.7	120 ± 18.9	123 ± 19.1	0.007
Diastolic blood pressure, mmHg	74.1 ± 12.6	72.6 ±12.2	73.1 ± 11.9	74.5 ± 13.4	76.1 ±12.4	0.0009
Platelet count, 10^9/L	235 ± 53.4	228 ± 53.8	235 ± 53.1	237 ± 51.1	241 ± 55.2	0.01
ALT, IU/l	18 (14–25)	18 (14–23)	17 (14–23)	18 (14–25)	19 (14–29)	< 0.0001
AST, IU/l	20 (17–24)	21 (19–25)	20 (17–24)	19 (17–23)	20 (17–24)	0.04
AST/ALT	1.15 ± 0.36	1.24 ± 0.35	1.20 ± 0.37	1.12 ± 0.33	1.04 ± 0.35	< 0.0001
APRI	0.25 (0.20–0.33)	0.28 (0.22–0.34)	0.25 (0.21–0.33)	0.24 (0.20–0.30)	0.24 (0.19–0.32)	0.0006
FIB-4	1.13 (0.82–1.52)	1.33 (0.98–1.76)	1.20 (0.89–1.59)	1.05 (0.76–1.38)	0.98 (0.70–1.36)	< 0.0001
GGT, IU/l	22 (16–33)	20 (15–31)	20 (16–30)	23 (16–34)	24 (17–38)	0.0009
C-reactive protein	0.4 (0.3–0.8)	0.3 (0.3–0.63)	0.4 (0.3–0.8)	0.4 (0.3–0.8)	0.5 (0.3–1.1)	< 0.0001
Amylase, IU/l	77.3 ± 24.9	110 ± 22.0	81.3 ± 4.7	66.6 ± 4.1	50.3 ± 7.0	—
(range)	(30–200)	(90–200)	(74–89)	(60–73)	(30–59)	
Triglyceride, mg/dl	88 (64–128)	83 (63–121)	84 (62–123)	88 (65–128)	100 (65–143)	0.001
HDL-cholesterol, mg/dl	61.2 ± 15.0	64.2 ± 15.4	62.6 ± 14.8	59.8 ± 14.6	58.3 ± 14.5	< 0.0001
Fasting plasma glucose, mg/dl	99.6 ± 16.7	97.5 ± 13.4	97.4 ± 11.4	101 ± 19.4	103 ± 20.4	< 0.0001
HbA1c (NGSP), %	5.7 ± 0.6	5.7 ± 0.5	5.7 ± 0.4	5.7 ± 0.6	5.8 ± 0.7	0.14
eGFR, ml/min/1.73m^2	75.0 ± 13.9	71.0 ± 14.3	74.3 ± 12.9	76.0 ± 14.2	78.7 ± 13.1	< 0.0001
ATP-III-Metabolic syndrome, n (%)	139 (9.4)	16 (4.3)	23 (6.2)	47 (12.3)	53 (15.0)	< 0.0001
Diabetes, n (%)	114 (7.7)	19 (5.1)	17 (4.6)	34 (8.9)	44 (12.4)	0.0002

NAFLD of all grades, n (%)	463 (31.4)	83 (22.5)	97 (26.1)	133 (34.9)	150 (42.4)	< 0.0001
Moderate to severe NAFLD, n (%)	221 (15.0)	34 (9.2)	42 (11.3)	60 (15.7)	85 (24.0)	< 0.0001
Medical history of						
Cardiovascular diseases, n (%)	55 (3.7)	11 (3.0)	20 (5.4)	11 (2.9)	13 (3.7)	0.24
Stroke, n (%)	26 (1.8)	12 (3.3)	6 (1.6)	4 (1.1)	4 (1.1)	0.08
Medications for						
Hypertension, n (%)	237 (16.1)	64 (17.3)	47 (12.7)	67 (17.6)	59 (16.7)	0.22
Hypercholesterolemia, n (%)	177 (12.0)	56 (15.2)	43 (11.6)	34 (8.9)	44 (12.4)	0.07
Diabetes, n (%)	56 (3.8)	12 (3.3)	12 (3.2)	15 (3.9)	17 (4.8)	0.65
Current smoker, n (%)	273 (18.5)	40 (10.8)	50 (13.5)	86 (22.6)	97 (27.4)	< 0.0001
Everyday alcohol consumers, n (%)	229 (15.5)	51 (13.8)	59 (15.9)	66 (17.3)	53 (15.0)	0.61
Regular exercise, n (%)	507 (34.4)	144 (39.0)	135 (36.4)	125 (32.9)	103 (29.1)	0.03

Data are means ±SD and medians (interquartile range). *P*-values for continuous variables and categorical variables were determined by analysis of variance and the χ^2 test, respectively. Regular exercise: ≥ 30 min exercise per session at least twice a week.

Table 2. Odds ratios for NAFLD-AG according to serum amylase quartiles

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
n	369	371	381	354	
Model 1	1	1.22 (0.87–1.71)	1.85 (1.34–2.55)	2.53 (1.84–3.50)	< 0.0001
Model 2	1	1.28 (0.91–1.81)	1.96 (1.39–2.74)	2.74 (1.95–3.85)	< 0.0001
Model 3	1	1.35 (0.93–1.97)	2.03 (1.41–2.94)	2.41 (1.66–3.51)	< 0.0001
Model 4	1	1.25 (0.86–1.82)	1.83 (1.26–2.65)	2.18 (1.49–3.18)	< 0.0001
Model 5	1	1.31 (0.90–1.90)	2.00 (1.39–2.89)	2.36 (1.62–3.44)	< 0.0001
Model 6	1	0.99 (0.66–1.49)	1.34 (0.89–2.01)	1.42 (0.94–2.15)	0.04
Model 7	1	1.02 (0.68–1.53)	1.30 (0.87–1.94)	1.49 (0.99–2.24)	0.03

Model 1: unadjusted

Model 2: adjusted for age, sex, and current smoking (*versus* non-smokers)

Model 3: Model 2 plus adjustment for log-transformed ALT, regular exercise (*versus* infrequent exercise), daily alcohol consumption (*versus* infrequent/no alcohol consumption), eGFR, past history of heart diseases and stroke, and medications for hypertension, diabetes, and dyslipidemia.

Model 4: Model 3 plus adjustment for diabetes, but excluding medications for hypertension, diabetes, and dyslipidemia.

Model 5: Model 3 plus adjustment for metabolic syndrome, but excluding medications for hypertension, diabetes, and dyslipidemia.

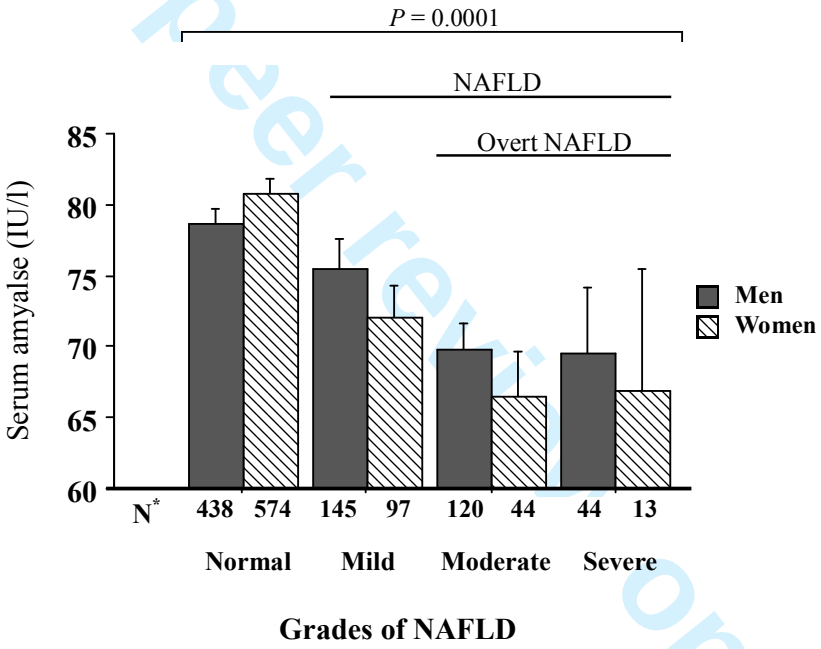
Model 6: Model 3 plus adjustment for BMI

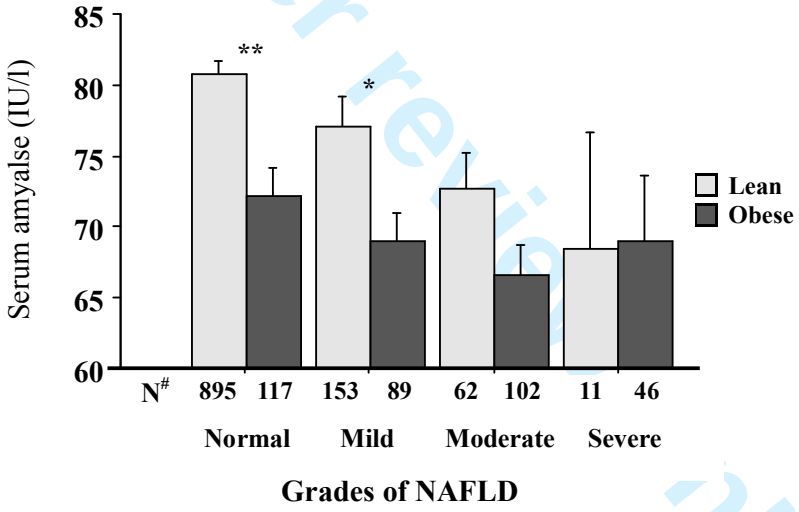
Model 7: Model 3 plus adjustment for WC

Table 3. Odds ratios of each serum amylase for NAFLD of moderate or severe grade

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
N*	320	316	308	289	
Model 1	1	1.29 (0.80–2.09)	2.04 (1.29–3.20)	3.51 (2.27–5.42)	< 0.0001
Model 2	1	1.36 (0.83–2.22)	2.14 (1.33–3.44)	3.81 (2.40–6.05)	< 0.0001
Model 3	1	1.61 (0.91–2.86)	2.32 (1.32–4.07)	3.45 (1.99–6.00)	< 0.0001
Model 4	1	1.48 (0.84–2.64)	2.11 (1.19–3.73)	2.97 (1.70–5.20)	< 0.0001
Model 5	1	1.56 (0.89–2.75)	2.32 (1.33–4.04)	3.26 (1.88–5.66)	< 0.0001
Model 6	1	1.05 (0.54–2.03)	1.44 (0.76–2.72)	2.01 (1.07–3.78)	0.01
Model 7	1	1.06 (0.55–2.05)	1.37 (0.73–2.59)	2.06 (1.09–3.87)	0.01

*Subjects with mild NAFLD (n = 242) were excluded from this analysis. Model 1 to Model 7 are the same as in **Table 2**.





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Independent association between low serum amylase and non-alcoholic fatty liver disease in asymptomatic adults: A cross-sectional observational study

Short title: Low serum amylase and non-alcoholic fatty liver disease

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ABSTRACT

Objectives: Low serum amylase (LSA) was reported to be associated with obesity, metabolic syndrome (MetS), and diabetes. However, it is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of MetS and insulin resistance. Therefore, we performed a clinical epidemiological study to investigate this potential association.

Design: A cross-sectional observational study with multivariate analysis.

Setting: Subjects were recruited in a healthcare centre in Saitama, an eastern district of Japan, near Tokyo.

Participants: A total of 1,475 asymptomatic adults aged 30–79 years who underwent detailed medical check-ups and who regularly consumed small amounts of alcohol (< 20 g/day).

Outcome measures: Serum amylase, cardiometabolic risk factors, NAFLD determined by ultrasound, MetS determined by Adult Treatment Panel-III criteria, and diabetes were assessed.

Results: The prevalence of NAFLD increased significantly from 22.5% to 42.4% (all grades) and from 9.2% to 24.0% (moderate or severe grade) from the highest to the lowest quartile of serum amylase. Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase, the lowest quartile of serum amylase was significantly associated with any-grade NAFLD and with moderate to

severe NAFLD, even after adjusting for MetS or diabetes. The association between LSA and any-grade NAFLD disappeared after further adjustment for body mass index or waist circumference, whereas the association between LSA and moderate or severe NAFLD remained statistically significant [odds ratios (95% confidence interval), 2.01 (1.07–3.78) and 2.06 (1.09–3.87), respectively, both $P = 0.01$].

Conclusions: Our results suggest that LSA may be associated with moderate or severe NAFLD in asymptomatic adults independent of MetS, diabetes, and obesity. These results warrant confirmation in further studies.

(274/300 words)

ARTICLE SUMMARY

Article focus

• The clinical relevance of low serum amylase (LSA) is unclear, but it is putatively associated with obesity-related metabolic abnormalities.

• It is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of cardiometabolic disease and insulin resistance.

Key messages

• Our results suggest that LSA is associated with NAFLD independent of metabolic syndrome, diabetes, and obesity.

• LSA may be an independent marker for moderate or severe NAFLD.

Strengths and limitations of this study

• A possible association between LSA and NAFLD was evaluated after fully adjusting for relevant confounding factors.

• NAFLD was diagnosed with ultrasound in this study. Other methods, such as computed tomography, magnetic resonance imaging, and fibroscan, may provide more precise assessment of NAFLD.

• This was an observational study and the cause–effect relationship is unknown.

INTRODUCTION

Abnormal serum amylase levels generally reflect overall dysfunction of the pancreas or salivary glands. Although the clinical relevance of elevated serum amylase levels has been extensively studied in relation to various conditions, including acute pancreatitis, pancreatic cancer, ectopic amylase-producing tumours, abdominal trauma, and kidney dysfunction [1-5], the clinical relevance of low serum amylase (LSA) has not been examined. LSA is conventionally considered as a crude marker for diffuse pancreas destruction secondary to pancreatic diseases, such as advanced chronic pancreatitis and cystic fibrosis [6,7]. LSA or low pancreatic amylase is also associated with insulin deficiency in patients with type 1 diabetes and in patients with longstanding type 2 diabetes [8-11]. In our recent community-based study, LSA, defined as a serum amylase concentration of < 60 IU/l, was observed in 25% of asymptomatic individuals [12]. LSA is also associated with metabolic syndrome (MetS), a cluster of cardiovascular risk factors, and diabetes [12,13]. However, obesity is thought to be the strongest predictor of LSA in the asymptomatic general population [12].

In the last two decades, there has been a marked increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide, along with an obesity pandemic [14,15]. NAFLD often progresses to a more severe condition, non-alcoholic steatohepatitis (NASH), and increased hepatic fibrosis is a significant histological feature of advanced NASH. NAFLD is considered to be a hepatic manifestation of

MetS and insulin resistance [14-18], which suggests that NAFLD consists of a wide spectrum of cardiometabolic diseases. It is also possible that NAFLD may reflect more pronounced insulin resistance compared with MetS in certain clinical settings, including in individuals without diabetes or obesity [17,19,20]. In this context, we hypothesised that LSA may be associated with NAFLD independently of MetS, type 2 diabetes, and even obesity. The aim of this study was to test this hypothesis and to investigate the clinical relevance of LSA, which is often observed in clinical practice. Therefore, we examined the associations among serum amylase, cardiometabolic risk factors, NAFLD, hepatic fibrosis, MetS, and diabetes in a cross-sectional study of asymptomatic adults.

METHODS

Protocol and subjects

The present report represents a series of observational studies performed in collaboration with Josai University, Sakado, Japan, and Social Insurance Omiya General Hospital that have been conducted to elucidate the relationships between lifestyle-related diseases and cardiometabolic risk factors. We recruited, at random, 2,472 asymptomatic subjects aged 30–79 years who underwent thorough annual medical check-ups, in which the subjects underwent a more extensive array of clinical tests than would be performed in routine check-ups, at the Social Insurance Omiya General Hospital, Saitama, Japan, between April 2009 and March 2010. The protocol

was approved by The Ethics Committee of Josai University and the Council of the Hospital, and informed consent was obtained from all participants.

The exclusion criteria and a flow chart summarizing subject disposition are shown in ***Supplemental Figure 1***. To exclude subjects with latent conditions likely to adversely affect the results, we excluded those with C-reactive protein ≥ 10.0 mg/l, estimated glomerular filtration rate (eGFR) ≤ 35 ml/min/1.73 m^2, serum amylase ≤ 30 IU/l (the lower 5^{th} percentile in an earlier study [12]) or ≥ 200 IU/l based on previous reports [21,22], as well as subjects suspected of having cancer. In the current analysis, to investigate the potential relationship between serum amylase and cardiometabolic features, including NAFLD, we included subjects with a wider range of serum amylase levels than the current reference ranges.

Subjects completed a questionnaire recording lifestyle factors, including habitual alcohol consumption, which was classified in terms of the frequency (none, occasional, and daily) and the amount of ethanol consumed per day (< 20 g, 20–39 g, 40–59 g, or ≥ 60 g). Subjects who habitually consumed ≥ 20 g ethanol per day were excluded from the study. Subjects positive for hepatic B virus surface antigen were also excluded. Hepatic C virus infection was not measured in this study. We also excluded subjects with other active liver diseases not examined, by excluded those with elevated serum liver enzyme levels (approximately three times the upper limit of normal): alanine aminotransferase (ALT) ≥ 150 IU/ml, aspartate aminotransferase (AST) ≥ 150 IU/ml, or

γ-glutamyltransferase (GGT) ≥ 150 IU/ml. Consequently, a total of 1,475 individuals were included in this study.

Laboratory measurements

Laboratory and anthropometric tests, and an abdominal ultrasound were performed in the early morning after an overnight fast. Serum parameters were measured using an AutoAnalyzer (Hitachi, Tokyo, Japan). The serum amylase level was measured using an enzymatic method (L-type Amylase; Wako, Tokyo, Japan) with a detection limit of 1.7 IU/l, and a run-to-run coefficient of variation of < 5.0%.

Abdominal ultrasound for the detection of fatty liver was carried out by registered medical sonographers who only work at Social Insurance Omiya General Hospital. The sonographers were blinded to the subjects' data. Fatty liver, which was determined by comparing liver echogenicity with that of the renal cortex [23], was defined as NAFLD. Additionally, the severity of NAFLD was graded into three categories: mild NAFLD, a slight increase in liver echogenicity with normal visualisation of the diaphragm and the portal veins; moderate NAFLD, a moderate increase in liver echogenicity with slightly impaired visualisation of the diaphragm and the portal veins; and severe NAFLD, a substantial increase in liver echogenicity with poor or no visualisation of the diaphragm and the portal veins [24,25]. In a previous study, the ultrasonographic steatosis score determined using these grades was highly correlated with the histological grade of

steatosis ($r = 0.80$, $P < 0.001$), but not with inflammatory activity ($r = 0.10$) or fibrosis score ($r = 0.19$) [25]. Therefore, the grade of NAFLD in this study probably reflects overall hepatic fat accumulation, rather than the severity of fibrosis. Since it is possible that mild NAFLD could include a normal liver, we defined overt NAFLD as moderate or severe NAFLD. We also examined the findings of gallstones, cholecystectomy, and splenomegaly, which was defined as a spleen index (calculated as the long dimension × short dimension on splenotomogram) ≥ 30 [26,27].

The liver fibrosis scores, the AST/ALT ratio [28] and FIB-4 were calculated in all patients, using a previously published formula:

FIB-4 = age (years) × AST/platelet count (10^9/l) × $ALT^{1/2}$) [29]

The diagnosis of MetS was based on the Adult Treatment Panel-III criteria [30] with the following cut-off limits: 1) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; 2) triglyceride ≥ 150 mg/dl; 3) high-density lipoprotein–cholesterol < 40 mg/dl for men and < 50 mg/dl for women; 4) fasting plasma glucose ≥ 100 mg/dl; and 5) waist circumference ≥ 90 cm for men and ≥ 80 cm for women. Subjects meeting three or more of these criteria, including treatment for any of these disorders, were defined as having MetS. Diabetes was defined as fasting plasma glucose ≥ 126 mg/dl or glycosylated haemoglobin (HbA1c) ≥ 6.5% (by the National Glycoprotein Standardization Program (NGSP)) according to the American Diabetes Association criteria [31], or treatment with oral hypoglycaemic drugs or insulin. HbA1c

(Japan Diabetes Society [JDS]) was converted to HbA1c (NGSP) units using the officially certified formula: HbA1c (NGSP) (%) = 1.02 × JDS (%) + 0.25% [32].

Since serum amylase can be affected by kidney function because of its excretion by the kidney [4,5], eGFR was considered as a confounding factor and was calculated using the following equation for Japanese subjects [33]: eGFR (ml/min/1.73 m^2) = 194 × serum $Cr^{-1.094}$ × $age^{-0.287}$ (if female) × 0.739. Here, Cr = serum creatinine concentration (mg/dl).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or median (interquartile range). Subjects were divided into quartiles according to serum amylase levels, where Q1 is the highest quartile and Q4 is the lowest quartile. *P*-values for continuous variables were determined using analysis of variance (ANOVA) or the Mann-Whitney *U* test, and for categorical variables using the χ^2 test. Highly skewed values (ALT, AST, GGT, and triglyceride) were log-transformed before analysis. Subjects were also stratified into four groups according to the grade of NAFLD (i.e., normal, mild, moderate, and severe NAFLD). Multivariate logistic regression models were used to examine the associations of quartiles (Q2–4) of serum amylase with any-grade NAFLD (NAFLD-AG), and with overt NAFLD relative to Q1, controlling for relevant confounding factors, including MetS, diabetes, and body mass index (BMI) or waist

circumference (WC). To elucidate any association between LSA and NAFLD, we examined the associations between LSA and NAFLD-AG, and between LSA and overt NAFLD. This analysis yielded odds ratios (OR) and 95% confidential intervals (95% CI). Tests for linear trends (*P* for trend) were calculated by treating the quartiles as a continuous variable (i.e., 1–4), and the same model analysis was conducted. Statistical analyses were performed using SPSS software version 18.0 (SPSS-IBM, Chicago, IL, USA) and Statview version 5.0 (SAS Institute, Cary, NC, USA). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The clinical characteristics of the subjects according to the quartile of serum amylase are shown in Table 1. The mean and median values of most clinical parameters, including platelet count and eGFR, increased significantly as serum amylase decreased, although there was a significant trend for subjects with lower amylase levels to be younger. No significant trend in HbA1c was observed against quartiles of serum amylase. The prevalence of NAFLD-AG and overt NAFLD increased significantly, from 22.5% to 42.4% and from 9.2% to 24%, respectively, from the highest (Q1) to the lowest serum amylase quartile (Q4). There were no significant differences in the prevalence rates of gallstones, cholecystectomy, or splenomegaly between quartiles, possibly because of the small numbers of subjects with these features, although the rates

of cholecystectomy and splenomegaly were higher in Q4 compared with the other quartiles. Log-transformed serum ALT and GGT increased significantly, whereas the AST/ALT ratio and FIB-4 decreased significantly with decreasing serum amylase quartile.

When subjects were stratified according to NAFLD grade, serum amylase decreased significantly with advancing grade of NAFLD (Figure 1). When subjects were further stratified according to sex, there was no significant difference between men and women. However, when the subjects were stratified according to high or low BMI (Figure 2), serum amylase levels were significantly lower in overweight/obese subjects (BMI $\geq$ 25.0 kg/m^2, mean ± SD BMI, 27.3 ± 2.3 kg/m^2, n = 1,121) than in lean subjects (BMI < 25.0 kg/m^2, mean ± SD BMI, 21.6 ± 2.0 kg/m^2, n = 354). Serum amylase levels in lean subjects decreased significantly with advancing grade of NAFLD, whereas those in obese subjects did not. Significant differences between lean and obese subjects were only observed in subjects with a normal liver or mild NAFLD. We did not conduct an analysis by further stratification according to MetS or diabetes because of the small proportion of subjects (both, < 10%).

Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase (≥ 90 IU/l, Q1), the lowest quartile (< 60 IU/l, Q4) was significantly associated with NAFLD-AG even after adjusting for confounders plus MetS or diabetes (Table 2, Models 4 and 5). In these conditions, MetS (OR: 3.66, 95%

CI: 2.37–5.65) and diabetes (OR: 1.96, 95% CI: 1.25–3.05) were significantly associated with Q4 of serum amylase (data not shown). However, these associations were markedly attenuated and were no longer statistically significant after adjusting for clinical confounders plus BMI or WC (Models 6 and 7), although there was a trend towards an association among the quartiles of serum amylase.

When we excluded subjects with mild NAFLD (n = 242) and repeated the analysis (Table 3), we detected stronger significant associations between Q4 and overt NAFLD. These associations were also moderately attenuated after additional adjustment for BMI or WC, but they remained statistically significant (Models 6 and 7). In this condition, BMI (OR: 1.65, 95% CI: 1.51–1.80) and WC (OR: 1.21, 95% CI: 1.17–1.25) were significantly associated with overt NAFLD (data not shown).

DISCUSSION

For the last few decades, NAFLD has been considered as a manifestation of MetS and insulin resistance [14-18]. In this context, the present study showed that LSA was significantly associated with NAFLD-AG in asymptomatic adults, independent of MetS, diabetes, and sex. Furthermore, LSA may be indicative of overt NAFLD, i.e., moderate or severe NAFLD, independent of obesity. These findings are not inconsistent because NAFLD may reflect insulin resistance, more so than MetS, in individuals without diabetes or obesity [17,19,20].

This study provided evidence that NAFLD can occur in lean individuals, who accounted for 37.8% (62/104) of subjects with moderate NAFLD and 19.3% (11/57) of subjects with severe NAFLD (Figure 2). This is consistent with the results of multivariate logistic analysis, where there was a significant trend for NAFLD against quartiles of serum amylase, and the significant association between the lowest quartile of serum amylase and NAFLD remained after adjusting for BMI and WC (Tables 2 and 3).

Potential mechanisms of the inverse relationship between serum amylase and NAFLD

We found no significant difference in serum amylase levels between lean and obese subjects with overt NAFLD, suggesting that the effects of obesity on serum amylase are apparent in subjects with a normal liver or with mild NAFLD, but not in subjects with overt NAFLD. Several studies have shown that NAFLD occurs in non-obese people, although the prevalence of NAFLD in non-obese subjects is lower than that in obese subjects [19,20,34]. Therefore, other than obesity, factors not examined in this study might also explain the association between LSA and NAFLD. Insulin resistance and obesity-related hormones, such as leptin, may be potential factors, because insulin resistance and energy imbalance are common pathophysiological findings in obese individuals [17,35]. Indeed, obesity plus insulin resistance is more closely associated

with metabolic abnormalities and NAFLD than is obesity without insulin resistance [17]. Hyperinsulinaemia caused by insulin resistance stimulates *de novo* lipogenesis in the liver through sterol regulatory element binding protein-1c (SREBP-1c) [36], irrespective of the influx of fatty acid from a meal and lipolysis in adipose tissues. Additionally, we recently reported a latent association between LSA and insulin resistance in a small study of asymptomatic middle-aged adults [37], supporting the hypothesis that LSA may be associated with NAFLD, at least in part through insulin resistance. However, because insulin resistance, as determined by the homeostasis model assessment of insulin resistance for example, was not examined in this study, further large epidemiological studies evaluating insulin resistance are needed to confirm the current hypothesis.

As another explanation for the inverse relationship between serum amylase and NAFLD, systemic ectopic fat deposition in organs including the pancreas (i.e., fatty pancreas) might contribute to the observed associations. This is because intrapancreatic fat, particularly intralobular pancreatic fat, was reported to be associated with NAFLD and MetS [38,39]. An animal study showed that obese mice had a heavier pancreas and more intrapancreatic fat [40]. Therefore, systemic fat deposits in multiple organs may be a common cause underlying the association between NAFLD and overall pancreatic dysfunction, which may result in impaired exocrine function, characterised as LSA.

Gene polymorphisms of patatin-like phospholipase domain containing 3

(PNPLA3) may also contribute to the strength of these associations. For example, a common variant of the PNPLA3 gene (rs738409) was reported to be associated with increased hepatic fat content (i.e., NAFLD) [41]. In the mouse liver, adiponutrin/PNPLA3 gene expression is under the direct transcriptional control of SREBP-1c, in response to insulin [42].

Serum amyalse and glucose metabolism

The prevalence of diabetes increased with decreasing serum amylase quartile in this study. However, similar to an earlier study [12], HbA1c was not associated with LSA, probably because most subjects in the lowest quartile of serum amylase in this study had no or only mild insulin resistance or mild hyperinsulinaemia, which is likely compensated for and results in euglycaemia or mild hyperglycaemia. In such conditions, fasting plasma glucose would increase linearly, as observed in Table 1, in response to insulin resistance, particularly in the early stage of diabetes [43,44].

Serum amyalse and hepatic fibrosis

To date, some markers have been considered for screening for NAFLD and mild hepatic fibrosis [45-47]. In this study, while the AST/ALT ratio and Fib-4 decreased significantly with the decreasing quartile of serum amylase, the platelet count increased (Table 1). Consequently, hepatic fibrosis is unlikely to be associated with NAFLD in

this study. A plausible explanation is that this study mostly consisted of non-obese individuals, with a small proportion having MetS and diabetes, resulting in a lower likelihood of advanced hepatic fibrosis and NASH. In fact, the estimated prevalence of NASH in this study is quite low when we compare it with the prevalence of NASH in a nationwide study in Japan. It was reported that approximately 20–25% of patients with diabetes had NAFLD, a population in which the prevalence of NASH might be 30–40% [48]. Therefore, the estimated prevalence of NASH might be less than 1% of all of the subjects in this study. In addition, the fact that more than half of the subjects in this study had repeatedly undergone detailed medical check-ups may also contribute to the lower prevalence of NASH, because repeated check-ups may promote consciousness of healthcare and favourable lifestyles.

Limitations

Several limitations should be mentioned. First, NAFLD was diagnosed by ultrasound rather than by histological examination, the gold standard technique for the diagnosis of NAFLD. Thus, NAFLD and the degree of hepatic fibrosis could be inaccurately evaluated, particularly in the early stages. In earlier studies, ultrasonography had a sensitivity of 60–94% and a specificity of 66–95% for detecting fatty liver [24,49]. However, its sensitivity is reduced in subjects with a small amount of fat (< 30%), such as those with mild NAFLD or advanced fibrosis, [14]. To improve the accuracy of

detecting and grading of NAFLD, the use of other imaging modalities might be needed, such as magnetic resonance imaging and magnetic resonance spectroscopy, which were reported to provide useful quantitative data in earlier studies [50,51].

Second, hepatitis C virus infection was not measured in this study. However, the prevalence of hepatitis C infection was reported to be 1.5–2.3% in Japan [52]. Low prevalence rates of hepatitis C (< 1.5%) were also recently reported in Asian-Pacific, tropical Latin American, and North American countries [53]. Therefore, hepatitis C infection is unlikely to profoundly modify the association between LSA and NAFLD in this study, although hepatitis C infection can contribute to the development of NAFLD [54]. It is also possible that individuals with primary biliary cirrhosis, autoimmune hepatitis or other forms of liver dysfunction (e.g., haemochromatosis and Wilson disease) were included in the present study, even though we excluded subjects with elevated hepatic enzymes (≥ 150 IU/ml). However, the prevalence of these diseases is quite low in the general population [55-57].

Third, because of the cross-sectional nature of the study, we could not determine the cause–effect relationship between LSA and NAFLD. However, in a previous retrospective study [12], subjects with LSA at baseline were more likely to develop MetS-related metabolic abnormalities 5 years later. Longitudinal prospective studies or clinical intervention trials are needed to elucidate the causality of the associations reported here.

Finally, the clinical relevance of measuring serum amylase remains unclear. The current results suggest that LSA is likely to detect overt NAFLD, but not NASH, which is the most important hepatic disorder. However, from the cardiometabolic perspective, even simple steatosis may be important, particularly in nonobese people, because steatosis may be more strongly associated with insulin resistance than is obesity [17,19,20]. Currently, serum amylase is rarely considered in clinical practice, except in certain situations, such as suspected pancreatitis. Therefore, numerous clinical and animal studies are needed to validate and confirm the clinical relevance of measuring serum amylase before it can be introduced into primary care for the detection of cardiometabolic diseases and NAFLD.

Conclusion

Our results suggest that LSA may be associated with NAFLD, particularly moderate or severe NAFLD, in asymptomatic adults. This association was independent of MetS, diabetes, and obesity. Further studies are needed to confirm the observed associations, and to explore the clinical relevance of LSA.

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Data sharing statement There are no additional data available.

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Figure Legends

Figure 1 Serum amylase levels according to the grade of NAFLD and sex. Serum amylase levels decreased significantly with advancing grade of NAFLD in both sexes (ANOVA, both $P = 0.0001$). There was no significant difference between men and women (two-way ANOVA, $P = 0.45$). *Number of subjects in each group. Bars represent standard errors.

Figure 2 Serum amylase levels according to the grade of NAFLD and obesity. Obesity was defined as BMI ≥ 25.0 kg/m^2. Serum amylase levels decreased significantly with advancing grade of NAFLD in lean subjects (ANOVA, $P = 0.01$), but not in obese subjects ($P = 0.33$). An overall significant difference was observed between lean and obese subjects (two-way ANOVA, $P = 0.03$). *Number of subjects in each group; $^{\dagger}P = 0.0001$ and $^{\ddagger}P = 0.02$ for lean versus obese subjects (Mann–Whitney U test). Bars represent standard errors.

Supplement Figure 1

Exclusion criteria of subjects and flow chart

Table 1. Characteristics of subjects stratified according to quartiles of serum amylase

	Total	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* value
n	1,475	369	371	381	354	
Age, y	55.1 ± 12.2	58.4 ± 11.8	56.4 ± 12.2	52.9 ± 11.7	52.7 ± 12.0	< 0.0001
Men, n (%)	747 (50.6)	178 (48.2)	176 (47.4)	205 (53.8)	188 (53.1)	0.19
BMI, kg/m^2	23.0 ± 3.2	21.9 ± 2.7	22.6 ± 3.2	23.3 ± 3.1	24.1 ± 3.4	< 0.0001
WC, cm	80.8 ± 8.9	78.2 ± 8.3	79.9 ± 8.8	81.8 ± 8.8	83.2 ± 9.1	< 0.0001
Systolic blood pressure, mmHg	120 ± 18.9	119 ± 18.5	119 ± 18.7	120 ± 18.9	123 ± 19.1	0.007
Diastolic blood pressure, mmHg	74.1 ± 12.6	72.6 ±12.2	73.1 ± 11.9	74.5 ± 13.4	76.1 ±12.4	0.0009
Platelet count, $10^9/L$	235 ± 53.4	228 ± 53.8	235 ± 53.1	237 ± 51.1	241 ± 55.2	0.01
ALT, IU/l	18 (14–25)	18 (14–23)	17 (14–23)	18 (14–25)	19 (14–29)	< 0.0001
AST, IU/l	20 (17–24)	21 (19–25)	20 (17–24)	19 (17–23)	20 (17–24)	0.04
AST/ALT	1.15 ± 0.36	1.24 ± 0.35	1.20 ± 0.37	1.12 ± 0.33	1.04 ± 0.35	< 0.0001

FIB-4	1.13 (0.82–1.52)	1.33 (0.98–1.76)	1.20 (0.89–1.59)	1.05 (0.76–1.38)	0.98 (0.70–1.36)	< 0.0001
GGT, IU/l	22 (16–33)	20 (15–31)	20 (16–30)	23 (16–34)	24 (17–38)	0.0009
C-reactive protein	0.4 (0.3–0.8)	0.3 (0.3–0.63)	0.4 (0.3–0.8)	0.4 (0.3–0.8)	0.5 (0.3–1.1)	< 0.0001
Amylase, IU/l	77.3 ± 24.9	110 ± 22.0	81.3 ± 4.7	66.6 ± 4.1	50.3 ± 7.0	—
(range)	(30–200)	(90–200)	(74–89)	(60–73)	(30–59)	
Triglyceride, mg/dl	88 (64–128)	83 (63–121)	84 (62–123)	88 (65–128)	100 (65–143)	0.001
HDL-cholesterol, mg/dl	61.2 ± 15.0	64.2 ± 15.4	62.6 ± 14.8	59.8 ± 14.6	58.3 ± 14.5	< 0.0001
Fasting plasma glucose, mg/dl	99.6 ± 16.7	97.5 ± 13.4	97.4 ± 11.4	101 ± 19.4	103 ± 20.4	< 0.0001
HbA1c (NGSP), %	5.7 ± 0.6	5.7 ± 0.5	5.7 ± 0.4	5.7 ± 0.6	5.8 ± 0.7	0.14
eGFR, ml/min/1.73m^2	75.0 ± 13.9	71.0 ± 14.3	74.3 ± 12.9	76.0 ± 14.2	78.7 ± 13.1	< 0.0001
ATP-III-Metabolic syndrome, n (%)	139 (9.4)	16 (4.3)	23 (6.2)	47 (12.3)	53 (15.0)	< 0.0001
Diabetes, n (%)	114 (7.7)	19 (5.1)	17 (4.6)	34 (8.9)	44 (12.4)	0.0002
NAFLD of all grades, n (%)	463 (31.4)	83 (22.5)	97 (26.1)	133 (34.9)	150 (42.4)	< 0.0001

Moderate to severe NAFLD, n (%)	221 (15.0)	34 (9.2)	42 (11.3)	60 (15.7)	85 (24.0)	< 0.0001
Gallstones, n (%)	81 (5.5)	21 (5.7)	20 (5.4)	24 (6.3)	16 (5.2)	0.76
Cholecystectomy, n (%)	23 (1.6)	5 (1.4)	3 (0.8)	5 (1.3)	10 (2.8)	0.15
Splenomegaly, n (%)	9 (0.6)	1 (0.3)	1 (0.3)	0	7 (2.0)	—*
Medical history of						
Cardiovascular diseases, n (%)	55 (3.7)	11 (3.0)	20 (5.4)	11 (2.9)	13 (3.7)	0.24
Stroke, n (%)	26 (1.8)	12 (3.3)	6 (1.6)	4 (1.1)	4 (1.1)	0.08
Medications for						
Hypertension, n (%)	237 (16.1)	64 (17.3)	47 (12.7)	67 (17.6)	59 (16.7)	0.22
Hypercholesterolemia, n (%)	177 (12.0)	56 (15.2)	43 (11.6)	34 (8.9)	44 (12.4)	0.07
Diabetes, n (%)	56 (3.8)	12 (3.3)	12 (3.2)	15 (3.9)	17 (4.8)	0.65
Current smoker, n (%)	273 (18.5)	40 (10.8)	50 (13.5)	86 (22.6)	97 (27.4)	< 0.0001
Everyday alcohol consumers, n (%)	229 (15.5)	51 (13.8)	59 (15.9)	66 (17.3)	53 (15.0)	0.61

Regular exercise, n (%)	507 (34.4)	144 (39.0)	135 (36.4)	125 (32.9)	103 (29.1)	0.03

Data are means ±SD and medians (interquartile range). *P*-values for continuous variables and categorical variables were determined by analysis of variance and the χ^2 test, respectively. Regular exercise: ≥ 30 min exercise per session at least twice a week.

*Statistical analysis was not performed because of the small number of subjects with splenomegaly.

Table 2. Odds ratios for NAFLD-AG according to serum amylase quartiles

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
n	369	371	381	354	
Model 1	1	1.22 (0.87–1.71)	1.85 (1.34–2.55)	2.53 (1.84–3.50)	< 0.0001
Model 2	1	1.28 (0.91–1.81)	1.96 (1.39–2.74)	2.74 (1.95–3.85)	< 0.0001
Model 3	1	1.35 (0.93–1.97)	2.03 (1.41–2.94)	2.41 (1.66–3.51)	< 0.0001
Model 4	1	1.25 (0.86–1.82)	1.83 (1.26–2.65)	2.18 (1.49–3.18)	< 0.0001
Model 5	1	1.31 (0.90–1.90)	2.00 (1.39–2.89)	2.36 (1.62–3.44)	< 0.0001
Model 6	1	0.99 (0.66–1.49)	1.34 (0.89–2.01)	1.42 (0.94–2.15)	0.04
Model 7	1	1.02 (0.68–1.53)	1.30 (0.87–1.94)	1.49 (0.99–2.24)	0.03

Model 1: unadjusted.

Model 2: adjusted for age, sex, and current smoking (*versus* non-smokers).

Model 3: Model 2 plus adjustment for log-transformed ALT, regular exercise (*versus* infrequent exercise), daily alcohol consumption (*versus* infrequent/no alcohol

consumption), eGFR, past history of heart diseases and stroke, and medications for hypertension, diabetes, and dyslipidemia.

Model 4: Model 3 plus adjustment for diabetes, but excluding medications for hypertension, diabetes, and dyslipidemia.

Model 5: Model 3 plus adjustment for metabolic syndrome, but excluding medications for hypertension, diabetes, and dyslipidemia.

Model 6: Model 3 plus adjustment for BMI.

Model 7: Model 3 plus adjustment for WC.

Table 3. Odds ratios of each serum amylase for NAFLD of moderate or severe grade

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
N*	320	316	308	289	
Model 1	1	1.29 (0.80–2.09)	2.04 (1.29–3.20)	3.51 (2.27–5.42)	< 0.0001
Model 2	1	1.36 (0.83–2.22)	2.14 (1.33–3.44)	3.81 (2.40–6.05)	< 0.0001
Model 3	1	1.61 (0.91–2.86)	2.32 (1.32–4.07)	3.45 (1.99–6.00)	< 0.0001
Model 4	1	1.48 (0.84–2.64)	2.11 (1.19–3.73)	2.97 (1.70–5.20)	< 0.0001
Model 5	1	1.56 (0.89–2.75)	2.32 (1.33–4.04)	3.26 (1.88–5.66)	< 0.0001
Model 6	1	1.05 (0.54–2.03)	1.44 (0.76–2.72)	2.01 (1.07–3.78)	0.01
Model 7	1	1.06 (0.55–2.05)	1.37 (0.73–2.59)	2.06 (1.09–3.87)	0.01

*Subjects with mild NAFLD (n = 242) were excluded from this analysis. Models 1–7 are the same as those in **Table 2**.

Independent association between low serum amylase and non-alcoholic fatty liver disease in asymptomatic adults: A cross-sectional observational study

Short title: Low serum amylase and non-alcoholic fatty liver disease

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Key words: serum amylase, NAFLD, metabolic syndrome, diabetes

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ABSTRACT

Objectives: Low serum amylase (LSA) was reported to be associated with obesity, metabolic syndrome (MetS), and diabetes. However, it is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of MetS and insulin resistance. Therefore, we performed a clinical epidemiological study to investigate this potential association.

Design: A cross-sectional observational study with multivariate analysis.

Setting: Subjects were recruited in a healthcare centre in Saitama, an eastern district of Japan, near Tokyo.

Participants: A total of 1,475 asymptomatic adults aged 30–79 years who underwent detailed medical check-ups and who regularly consumed small amounts of alcohol (< 20 g/day).

Outcome measures: Serum amylase, cardiometabolic risk factors, NAFLD determined by ultrasound, MetS determined by Adult Treatment Panel-III criteria, and diabetes were assessed.

Results: The prevalence of NAFLD increased significantly from 22.5% to 42.4% (all grades) and from 9.2% to 24.0% (moderate or severe grade) from the highest to the lowest quartile of serum amylase. Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase, the lowest quartile of serum amylase was significantly associated with any-grade NAFLD and with moderate to

severe NAFLD, even after adjusting for MetS or diabetes. The association between LSA and any-grade NAFLD disappeared after further adjustment for body mass index or waist circumference, whereas the association between LSA and moderate or severe NAFLD remained statistically significant [odds ratios (95% confidence interval), 2.01 (1.07–3.78) and 2.06 (1.09–3.87), respectively, both $P = 0.01$].

Conclusions: Our results suggest that LSA may be associated with moderate or severe NAFLD in asymptomatic adults independent of MetS, diabetes, and obesity. These results warrant confirmation in further studies.

(274/300 words)

ARTICLE SUMMARY

Article focus

• The clinical relevance of low serum amylase (LSA) is unclear, but it is putatively associated with obesity-related metabolic abnormalities.

• It is unknown whether LSA is associated with non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of cardiometabolic disease and insulin resistance.

Key messages

• Our results suggest that LSA is associated with NAFLD independent of metabolic syndrome, diabetes, and obesity.

• LSA may be an independent marker for moderate or severe NAFLD.

Strengths and limitations of this study

• A possible association between LSA and NAFLD was evaluated after fully adjusting for relevant confounding factors.

• NAFLD was diagnosed with ultrasound in this study. Other methods, such as computed tomography, magnetic resonance imaging, and fibroscan, may provide more precise assessment of NAFLD.

• This was an observational study and the cause–effect relationship is unknown.

INTRODUCTION

Abnormal serum amylase levels generally reflect overall dysfunction of the pancreas or salivary glands. Although the clinical relevance of elevated serum amylase levels has been extensively studied in relation to various conditions, including acute pancreatitis, pancreatic cancer, ectopic amylase-producing tumours, abdominal trauma, and kidney dysfunction [1-5], the clinical relevance of low serum amylase (LSA) has not been examined. LSA is conventionally considered as a crude marker for diffuse pancreas destruction secondary to pancreatic diseases, such as advanced chronic pancreatitis and cystic fibrosis [6,7]. LSA or low pancreatic amylase is also associated with insulin deficiency in patients with type 1 diabetes and in patients with longstanding type 2 diabetes [8-11]. In our recent community-based study, LSA, defined as a serum amylase concentration of < 60 IU/l, was observed in 25% of asymptomatic individuals [12]. LSA is also associated with metabolic syndrome (MetS), a cluster of cardiovascular risk factors, and diabetes [12,13]. However, obesity is thought to be the strongest predictor of LSA in the asymptomatic general population [12].

In the last two decades, there has been a marked increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide, along with an obesity pandemic [14,15]. NAFLD often progresses to a more severe condition, non-alcoholic steatohepatitis (NASH), and increased hepatic fibrosis is a significant histological feature of advanced NASH. NAFLD is considered to be a hepatic manifestation of

MetS and insulin resistance [14-18], which suggests that NAFLD consists of a wide spectrum of cardiometabolic diseases. It is also possible that NAFLD may reflect more pronounced insulin resistance compared with MetS in certain clinical settings, including in individuals without diabetes or obesity [17,19,20]. In this context, we hypothesised that LSA may be associated with NAFLD independently of MetS, type 2 diabetes, and even obesity. The aim of this study was to test this hypothesis and to investigate the clinical relevance of LSA, which is often observed in clinical practice. Therefore, we examined the associations among serum amylase, cardiometabolic risk factors, NAFLD, hepatic fibrosis, MetS, and diabetes in a cross-sectional study of asymptomatic adults.

METHODS

Protocol and subjects

The present report represents a series of observational studies performed in collaboration with Josai University, Sakado, Japan, and Social Insurance Omiya General Hospital that have been conducted to elucidate the relationships between lifestyle-related diseases and cardiometabolic risk factors. We recruited, at random, 2,472 asymptomatic subjects aged 30–79 years who underwent thorough annual medical check-ups, in which the subjects underwent a more extensive array of clinical tests than would be performed in routine check-ups, at the Social Insurance Omiya General Hospital, Saitama, Japan, between April 2009 and March 2010. The protocol

was approved by The Ethics Committee of Josai University and the Council of the Hospital, and informed consent was obtained from all participants.

The exclusion criteria and a flow chart summarizing subject disposition are shown in ***Supplemental Figure 1***. To exclude subjects with latent conditions likely to adversely affect the results, we excluded those with C-reactive protein ≥ 10.0 mg/l, estimated glomerular filtration rate (eGFR) ≤ 35 ml/min/1.73 m^2, serum amylase ≤ 30 IU/l (the lower 5th percentile in an earlier study [12]) or ≥ 200 IU/l based on previous reports [21,22], as well as subjects suspected of having cancer. In the current analysis, to investigate the potential relationship between serum amylase and cardiometabolic features, including NAFLD, we included subjects with a wider range of serum amylase levels than the current reference ranges.

Subjects completed a questionnaire recording lifestyle factors, including habitual alcohol consumption, which was classified in terms of the frequency (none, occasional, and daily) and the amount of ethanol consumed per day (< 20 g, 20–39 g, 40–59 g, or ≥ 60 g). Subjects who habitually consumed ≥ 20 g ethanol per day were excluded from the study. Subjects positive for hepatic B virus surface antigen were also excluded. Hepatic C virus infection was not measured in this study. We also excluded subjects with other active liver diseases not examined, by excluded those with elevated serum liver enzyme levels (approximately three times the upper limit of normal): alanine aminotransferase (ALT) ≥ 150 IU/ml, aspartate aminotransferase (AST) ≥ 150 IU/ml, or

γ-glutamyltransferase (GGT) ≥ 150 IU/ml. Consequently, a total of 1,475 individuals were included in this study.

Laboratory measurements

Laboratory and anthropometric tests, and an abdominal ultrasound were performed in the early morning after an overnight fast. Serum parameters were measured using an AutoAnalyzer (Hitachi, Tokyo, Japan). The serum amylase level was measured using an enzymatic method (L-type Amylase; Wako, Tokyo, Japan) with a detection limit of 1.7 IU/l, and a run-to-run coefficient of variation of < 5.0%.

Abdominal ultrasound for the detection of fatty liver was carried out by registered medical sonographers who only work at Social Insurance Omiya General Hospital. The sonographers were blinded to the subjects' data. Fatty liver, which was determined by comparing liver echogenicity with that of the renal cortex [23], was defined as NAFLD. Additionally, the severity of NAFLD was graded into three categories: mild NAFLD, a slight increase in liver echogenicity with normal visualisation of the diaphragm and the portal veins; moderate NAFLD, a moderate increase in liver echogenicity with slightly impaired visualisation of the diaphragm and the portal veins; and severe NAFLD, a substantial increase in liver echogenicity with poor or no visualisation of the diaphragm and the portal veins [24,25]. In a previous study, the ultrasonographic steatosis score determined using these grades was highly correlated with the histological grade of

steatosis ($r = 0.80$, $P < 0.001$), but not with inflammatory activity ($r = 0.10$) or fibrosis score ($r = 0.19$) [25]. Therefore, the grade of NAFLD in this study probably reflects overall hepatic fat accumulation, rather than the severity of fibrosis. Since it is possible that mild NAFLD could include a normal liver, we defined overt NAFLD as moderate or severe NAFLD. We also examined the findings of gallstones, cholecystectomy, and splenomegaly, which was defined as a spleen index (calculated as the long dimension × short dimension on splenotomogram) ≥ 30 [26,27].

The liver fibrosis scores, the AST/ALT ratio [28] and FIB-4 were calculated in all patients, using a previously published formula:

FIB-4 = age (years) × AST/platelet count (10^9/l) × $ALT^{1/2}$) [29]

The diagnosis of MetS was based on the Adult Treatment Panel-III criteria [30] with the following cut-off limits: 1) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; 2) triglyceride ≥ 150 mg/dl; 3) high-density lipoprotein–cholesterol < 40 mg/dl for men and < 50 mg/dl for women; 4) fasting plasma glucose ≥ 100 mg/dl; and 5) waist circumference ≥ 90 cm for men and ≥ 80 cm for women. Subjects meeting three or more of these criteria, including treatment for any of these disorders, were defined as having MetS. Diabetes was defined as fasting plasma glucose ≥ 126 mg/dl or glycosylated haemoglobin (HbA1c) ≥ 6.5% (by the National Glycoprotein Standardization Program (NGSP)) according to the American Diabetes Association criteria [31], or treatment with oral hypoglycaemic drugs or insulin. HbA1c

(Japan Diabetes Society [JDS]) was converted to HbA1c (NGSP) units using the officially certified formula: HbA1c (NGSP) (%) = 1.02 × JDS (%) + 0.25% [32].

Since serum amylase can be affected by kidney function because of its excretion by the kidney [4,5], eGFR was considered as a confounding factor and was calculated using the following equation for Japanese subjects [33]: eGFR (ml/min/1.73 m^2) = 194 × serum $Cr^{-1.094}$ × $age^{-0.287}$ (if female) × 0.739. Here, Cr = serum creatinine concentration (mg/dl).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or median (interquartile range). Subjects were divided into quartiles according to serum amylase levels, where Q1 is the highest quartile and Q4 is the lowest quartile. *P*-values for continuous variables were determined using analysis of variance (ANOVA) or the Mann-Whitney *U* test, and for categorical variables using the χ^2 test. Highly skewed values (ALT, AST, GGT, and triglyceride) were log-transformed before analysis. Subjects were also stratified into four groups according to the grade of NAFLD (i.e., normal, mild, moderate, and severe NAFLD). Multivariate logistic regression models were used to examine the associations of quartiles (Q2–4) of serum amylase with any-grade NAFLD (NAFLD-AG), and with overt NAFLD relative to Q1, controlling for relevant confounding factors, including MetS, diabetes, and body mass index (BMI) or waist

circumference (WC). To elucidate any association between LSA and NAFLD, we examined the associations between LSA and NAFLD-AG, and between LSA and overt NAFLD. This analysis yielded odds ratios (OR) and 95% confidential intervals (95% CI). Tests for linear trends (*P* for trend) were calculated by treating the quartiles as a continuous variable (i.e., 1–4), and the same model analysis was conducted. Statistical analyses were performed using SPSS software version 18.0 (SPSS-IBM, Chicago, IL, USA) and Statview version 5.0 (SAS Institute, Cary, NC, USA). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The clinical characteristics of the subjects according to the quartile of serum amylase are shown in Table 1. The mean and median values of most clinical parameters, including platelet count and eGFR, increased significantly as serum amylase decreased, although there was a significant trend for subjects with lower amylase levels to be younger. No significant trend in HbA1c was observed against quartiles of serum amylase. The prevalence of NAFLD-AG and overt NAFLD increased significantly, from 22.5% to 42.4% and from 9.2% to 24%, respectively, from the highest (Q1) to the lowest serum amylase quartile (Q4). There were no significant differences in the prevalence rates of gallstones, cholecystectomy, or splenomegaly between quartiles, possibly because of the small numbers of subjects with these features, although the rates

of cholecystectomy and splenomegaly were higher in Q4 compared with the other quartiles. Log-transformed serum ALT and GGT increased significantly, whereas the AST/ALT ratio and FIB-4 decreased significantly with decreasing serum amylase quartile.

When subjects were stratified according to NAFLD grade, serum amylase decreased significantly with advancing grade of NAFLD (Figure 1). When subjects were further stratified according to sex, there was no significant difference between men and women. However, when the subjects were stratified according to high or low BMI (Figure 2), serum amylase levels were significantly lower in overweight/obese subjects (BMI $\geq$ 25.0 kg/m^2, mean ± SD BMI, 27.3 ± 2.3 kg/m^2, n = 1,121) than in lean subjects (BMI < 25.0 kg/m^2, mean ± SD BMI, 21.6 ± 2.0 kg/m^2, n = 354). Serum amylase levels in lean subjects decreased significantly with advancing grade of NAFLD, whereas those in obese subjects did not. Significant differences between lean and obese subjects were only observed in subjects with a normal liver or mild NAFLD. We did not conduct an analysis by further stratification according to MetS or diabetes because of the small proportion of subjects (both, < 10%).

Multiple logistic regression analysis showed that, compared with the highest quartile of serum amylase ($\geq$ 90 IU/l, Q1), the lowest quartile (< 60 IU/l, Q4) was significantly associated with NAFLD-AG even after adjusting for confounders plus MetS or diabetes (Table 2, Models 4 and 5). In these conditions, MetS (OR: 3.66, 95%

CI: 2.37–5.65) and diabetes (OR: 1.96, 95% CI: 1.25–3.05) were significantly associated with Q4 of serum amylase (data not shown). However, these associations were markedly attenuated and were no longer statistically significant after adjusting for clinical confounders plus BMI or WC (Models 6 and 7), although there was a trend towards an association among the quartiles of serum amylase.

When we excluded subjects with mild NAFLD (n = 242) and repeated the analysis (Table 3), we detected stronger significant associations between Q4 and overt NAFLD. These associations were also moderately attenuated after additional adjustment for BMI or WC, but they remained statistically significant (Models 6 and 7). In this condition, BMI (OR: 1.65, 95% CI: 1.51–1.80) and WC (OR: 1.21, 95% CI: 1.17–1.25) were significantly associated with overt NAFLD (data not shown).

DISCUSSION

For the last few decades, NAFLD has been considered as a manifestation of MetS and insulin resistance [14-18]. In this context, the present study showed that LSA was significantly associated with NAFLD-AG in asymptomatic adults, independent of MetS, diabetes, and sex. Furthermore, LSA may be indicative of overt NAFLD, i.e., moderate or severe NAFLD, independent of obesity. These findings are not inconsistent because NAFLD may reflect insulin resistance, more so than MetS, in individuals without diabetes or obesity [17,19,20].

This study provided evidence that NAFLD can occur in lean individuals, who accounted for 37.8% (62/104) of subjects with moderate NAFLD and 19.3% (11/57) of subjects with severe NAFLD (Figure 2). This is consistent with the results of multivariate logistic analysis, where there was a significant trend for NAFLD against quartiles of serum amylase, and the significant association between the lowest quartile of serum amylase and NAFLD remained after adjusting for BMI and WC (Tables 2 and 3).

Potential mechanisms of the inverse relationship between serum amylase and NAFLD

We found no significant difference in serum amylase levels between lean and obese subjects with overt NAFLD, suggesting that the effects of obesity on serum amylase are apparent in subjects with a normal liver or with mild NAFLD, but not in subjects with overt NAFLD. Several studies have shown that NAFLD occurs in non-obese people, although the prevalence of NAFLD in non-obese subjects is lower than that in obese subjects [19,20,34]. Therefore, other than obesity, factors not examined in this study might also explain the association between LSA and NAFLD. Insulin resistance and obesity-related hormones, such as leptin, may be potential factors, because insulin resistance and energy imbalance are common pathophysiological findings in obese individuals [17,35]. Indeed, obesity plus insulin resistance is more closely associated

with metabolic abnormalities and NAFLD than is obesity without insulin resistance [17]. Hyperinsulinaemia caused by insulin resistance stimulates *de novo* lipogenesis in the liver through sterol regulatory element binding protein-1c (SREBP-1c) [36], irrespective of the influx of fatty acid from a meal and lipolysis in adipose tissues. Additionally, we recently reported a latent association between LSA and insulin resistance in a small study of asymptomatic middle-aged adults [37], supporting the hypothesis that LSA may be associated with NAFLD, at least in part through insulin resistance. However, because insulin resistance, as determined by the homeostasis model assessment of insulin resistance for example, was not examined in this study, further large epidemiological studies evaluating insulin resistance are needed to confirm the current hypothesis.

As another explanation for the inverse relationship between serum amylase and NAFLD, systemic ectopic fat deposition in organs including the pancreas (i.e., fatty pancreas) might contribute to the observed associations. This is because intrapancreatic fat, particularly intralobular pancreatic fat, was reported to be associated with NAFLD and MetS [38,39]. An animal study showed that obese mice had a heavier pancreas and more intrapancreatic fat [40]. Therefore, systemic fat deposits in multiple organs may be a common cause underlying the association between NAFLD and overall pancreatic dysfunction, which may result in impaired exocrine function, characterised as LSA.

Gene polymorphisms of patatin-like phospholipase domain containing 3

(PNPLA3) may also contribute to the strength of these associations. For example, a common variant of the PNPLA3 gene (rs738409) was reported to be associated with increased hepatic fat content (i.e., NAFLD) [41]. In the mouse liver, adiponutrin/PNPLA3 gene expression is under the direct transcriptional control of SREBP-1c, in response to insulin [42].

Serum amyalse and glucose metabolism

The prevalence of diabetes increased with decreasing serum amylase quartile in this study. However, similar to an earlier study [12], HbA1c was not associated with LSA, probably because most subjects in the lowest quartile of serum amylase in this study had no or only mild insulin resistance or mild hyperinsulinaemia, which is likely compensated for and results in euglycaemia or mild hyperglycaemia. In such conditions, fasting plasma glucose would increase linearly, as observed in Table 1, in response to insulin resistance, particularly in the early stage of diabetes [43,44].

Serum amyalse and hepatic fibrosis

To date, some markers have been considered for screening for NAFLD and mild hepatic fibrosis [45-47]. In this study, while the AST/ALT ratio and Fib-4 decreased significantly with the decreasing quartile of serum amylase, the platelet count increased (Table 1). Consequently, hepatic fibrosis is unlikely to be associated with NAFLD in

this study. A plausible explanation is that this study mostly consisted of non-obese individuals, with a small proportion having MetS and diabetes, resulting in a lower likelihood of advanced hepatic fibrosis and NASH. In fact, the estimated prevalence of NASH in this study is quite low when we compare it with the prevalence of NASH in a nationwide study in Japan. It was reported that approximately 20–25% of patients with diabetes had NAFLD, a population in which the prevalence of NASH might be 30–40% [48]. Therefore, the estimated prevalence of NASH might be less than 1% of all of the subjects in this study. In addition, the fact that more than half of the subjects in this study had repeatedly undergone detailed medical check-ups may also contribute to the lower prevalence of NASH, because repeated check-ups may promote consciousness of healthcare and favourable lifestyles.

Limitations

Several limitations should be mentioned. First, NAFLD was diagnosed by ultrasound rather than by histological examination, the gold standard technique for the diagnosis of NAFLD. Thus, NAFLD and the degree of hepatic fibrosis could be inaccurately evaluated, particularly in the early stages. In earlier studies, ultrasonography had a sensitivity of 60–94% and a specificity of 66–95% for detecting fatty liver [24,49]. However, its sensitivity is reduced in subjects with a small amount of fat (< 30%), such as those with mild NAFLD or advanced fibrosis, [14]. To improve the accuracy of

detecting and grading of NAFLD, the use of other imaging modalities might be needed, such as magnetic resonance imaging and magnetic resonance spectroscopy, which were reported to provide useful quantitative data in earlier studies [50,51].

Second, hepatitis C virus infection was not measured in this study. However, the prevalence of hepatitis C infection was reported to be 1.5–2.3% in Japan [52]. Low prevalence rates of hepatitis C (< 1.5%) were also recently reported in Asian-Pacific, tropical Latin American, and North American countries [53]. Therefore, hepatitis C infection is unlikely to profoundly modify the association between LSA and NAFLD in this study, although hepatitis C infection can contribute to the development of NAFLD [54]. It is also possible that individuals with primary biliary cirrhosis, autoimmune hepatitis or other forms of liver dysfunction (e.g., haemochromatosis and Wilson disease) were included in the present study, even though we excluded subjects with elevated hepatic enzymes (≥ 150 IU/ml). However, the prevalence of these diseases is quite low in the general population [55-57].

Third, because of the cross-sectional nature of the study, we could not determine the cause–effect relationship between LSA and NAFLD. However, in a previous retrospective study [12], subjects with LSA at baseline were more likely to develop MetS-related metabolic abnormalities 5 years later. Longitudinal prospective studies or clinical intervention trials are needed to elucidate the causality of the associations reported here.

Finally, the clinical relevance of measuring serum amylase remains unclear. The current results suggest that LSA is likely to detect overt NAFLD, but not NASH, which is the most important hepatic disorder. However, from the cardiometabolic perspective, even simple steatosis may be important, particularly in nonobese people, because steatosis may be more strongly associated with insulin resistance than is obesity [17,19,20]. Currently, serum amylase is rarely considered in clinical practice, except in certain situations, such as suspected pancreatitis. Therefore, numerous clinical and animal studies are needed to validate and confirm the clinical relevance of measuring serum amylase before it can be introduced into primary care for the detection of cardiometabolic diseases and NAFLD.

Conclusion

Our results suggest that LSA may be associated with NAFLD, particularly moderate or severe NAFLD, in asymptomatic adults. This association was independent of MetS, diabetes, and obesity. Further studies are needed to confirm the observed associations, and to explore the clinical relevance of LSA.

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Figure Legends

Figure 1 Serum amylase levels according to the grade of NAFLD and sex. Serum amylase levels decreased significantly with advancing grade of NAFLD in both sexes (ANOVA, both $P = 0.0001$). There was no significant difference between men and women (two-way ANOVA, $P = 0.45$). *Number of subjects in each group. Bars represent standard errors.

Figure 2 Serum amylase levels according to the grade of NAFLD and obesity. Obesity was defined as BMI ≥ 25.0 kg/m^2. Serum amylase levels decreased significantly with advancing grade of NAFLD in lean subjects (ANOVA, $P = 0.01$), but not in obese subjects ($P = 0.33$). An overall significant difference was observed between lean and obese subjects (two-way ANOVA, $P = 0.03$). *Number of subjects in each group; $^{\dagger}P = 0.0001$ and $^{\ddagger}P = 0.02$ for lean versus obese subjects (Mann–Whitney U test). Bars represent standard errors.

Supplement Figure 1

Exclusion criteria of subjects and flow chart

Table 1. Characteristics of subjects stratified according to quartiles of serum amylase

	Total	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* value
n	1,475	369	371	381	354	
Age, y	55.1 ± 12.2	58.4 ± 11.8	56.4 ± 12.2	52.9 ± 11.7	52.7 ± 12.0	< 0.0001
Men, n (%)	747 (50.6)	178 (48.2)	176 (47.4)	205 (53.8)	188 (53.1)	0.19
BMI, kg/m^2	23.0 ± 3.2	21.9 ± 2.7	22.6 ± 3.2	23.3 ± 3.1	24.1 ± 3.4	< 0.0001
WC, cm	80.8 ± 8.9	78.2 ± 8.3	79.9 ± 8.8	81.8 ± 8.8	83.2 ± 9.1	< 0.0001
Systolic blood pressure, mmHg	120 ± 18.9	119 ± 18.5	119 ± 18.7	120 ± 18.9	123 ± 19.1	0.007
Diastolic blood pressure, mmHg	74.1 ± 12.6	72.6 ±12.2	73.1 ± 11.9	74.5 ± 13.4	76.1 ±12.4	0.0009
Platelet count, $10^9/L$	235 ± 53.4	228 ± 53.8	235 ± 53.1	237 ± 51.1	241 ± 55.2	0.01
ALT, IU/l	18 (14–25)	18 (14–23)	17 (14–23)	18 (14–25)	19 (14–29)	< 0.0001
AST, IU/l	20 (17–24)	21 (19–25)	20 (17–24)	19 (17–23)	20 (17–24)	0.04
AST/ALT	1.15 ± 0.36	1.24 ± 0.35	1.20 ± 0.37	1.12 ± 0.33	1.04 ± 0.35	< 0.0001

FIB-4	1.13 (0.82–1.52)	1.33 (0.98–1.76)	1.20 (0.89–1.59)	1.05 (0.76–1.38)	0.98 (0.70–1.36)	< 0.0001
GGT, IU/l	22 (16–33)	20 (15–31)	20 (16–30)	23 (16–34)	24 (17–38)	0.0009
C-reactive protein	0.4 (0.3–0.8)	0.3 (0.3–0.63)	0.4 (0.3–0.8)	0.4 (0.3–0.8)	0.5 (0.3–1.1)	< 0.0001
Amylase, IU/l	77.3 ± 24.9	110 ± 22.0	81.3 ± 4.7	66.6 ± 4.1	50.3 ± 7.0	—
(range)	(30–200)	(90–200)	(74–89)	(60–73)	(30–59)	
Triglyceride, mg/dl	88 (64–128)	83 (63–121)	84 (62–123)	88 (65–128)	100 (65–143)	0.001
HDL-cholesterol, mg/dl	61.2 ± 15.0	64.2 ± 15.4	62.6 ± 14.8	59.8 ± 14.6	58.3 ± 14.5	< 0.0001
Fasting plasma glucose, mg/dl	99.6 ± 16.7	97.5 ± 13.4	97.4 ± 11.4	101 ± 19.4	103 ± 20.4	< 0.0001
HbA1c (NGSP), %	5.7 ± 0.6	5.7 ± 0.5	5.7 ± 0.4	5.7 ± 0.6	5.8 ± 0.7	0.14
eGFR, ml/min/1.73m^2	75.0 ± 13.9	71.0 ± 14.3	74.3 ± 12.9	76.0 ± 14.2	78.7 ± 13.1	< 0.0001
ATP-III-Metabolic syndrome, n (%)	139 (9.4)	16 (4.3)	23 (6.2)	47 (12.3)	53 (15.0)	< 0.0001
Diabetes, n (%)	114 (7.7)	19 (5.1)	17 (4.6)	34 (8.9)	44 (12.4)	0.0002
NAFLD of all grades, n (%)	463 (31.4)	83 (22.5)	97 (26.1)	133 (34.9)	150 (42.4)	< 0.0001

Moderate to severe NAFLD, n (%)	221 (15.0)	34 (9.2)	42 (11.3)	60 (15.7)	85 (24.0)	< 0.0001
Gallstones, n (%)	81 (5.5)	21 (5.7)	20 (5.4)	24 (6.3)	16 (5.2)	0.76
Cholecystectomy, n (%)	23 (1.6)	5 (1.4)	3 (0.8)	5 (1.3)	10 (2.8)	0.15
Splenomegaly, n (%)	9 (0.6)	1 (0.3)	1 (0.3)	0	7 (2.0)	—*
Medical history of						
Cardiovascular diseases, n (%)	55 (3.7)	11 (3.0)	20 (5.4)	11 (2.9)	13 (3.7)	0.24
Stroke, n (%)	26 (1.8)	12 (3.3)	6 (1.6)	4 (1.1)	4 (1.1)	0.08
Medications for						
Hypertension, n (%)	237 (16.1)	64 (17.3)	47 (12.7)	67 (17.6)	59 (16.7)	0.22
Hypercholesterolemia, n (%)	177 (12.0)	56 (15.2)	43 (11.6)	34 (8.9)	44 (12.4)	0.07
Diabetes, n (%)	56 (3.8)	12 (3.3)	12 (3.2)	15 (3.9)	17 (4.8)	0.65
Current smoker, n (%)	273 (18.5)	40 (10.8)	50 (13.5)	86 (22.6)	97 (27.4)	< 0.0001
Everyday alcohol consumers, n (%)	229 (15.5)	51 (13.8)	59 (15.9)	66 (17.3)	53 (15.0)	0.61

Regular exercise, n (%)	507 (34.4)	144 (39.0)	135 (36.4)	125 (32.9)	103 (29.1)	0.03

Data are means ±SD and medians (interquartile range). *P*-values for continuous variables and categorical variables were determined by analysis of variance and the χ^2 test, respectively. Regular exercise: ≥ 30 min exercise per session at least twice a week.

*Statistical analysis was not performed because of the small number of subjects with splenomegaly.

Table 2. Odds ratios for NAFLD-AG according to serum amylase quartiles

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
n	369	371	381	354	
Model 1	1	1.22 (0.87–1.71)	1.85 (1.34–2.55)	2.53 (1.84–3.50)	< 0.0001
Model 2	1	1.28 (0.91–1.81)	1.96 (1.39–2.74)	2.74 (1.95–3.85)	< 0.0001
Model 3	1	1.35 (0.93–1.97)	2.03 (1.41–2.94)	2.41 (1.66–3.51)	< 0.0001
Model 4	1	1.25 (0.86–1.82)	1.83 (1.26–2.65)	2.18 (1.49–3.18)	< 0.0001
Model 5	1	1.31 (0.90–1.90)	2.00 (1.39–2.89)	2.36 (1.62–3.44)	< 0.0001
Model 6	1	0.99 (0.66–1.49)	1.34 (0.89–2.01)	1.42 (0.94–2.15)	0.04
Model 7	1	1.02 (0.68–1.53)	1.30 (0.87–1.94)	1.49 (0.99–2.24)	0.03

Model 1: unadjusted.

Model 2: adjusted for age, sex, and current smoking (*versus* non-smokers).

Model 3: Model 2 plus adjustment for log-transformed ALT, regular exercise (*versus* infrequent exercise), daily alcohol consumption (*versus* infrequent/no alcohol

consumption), eGFR, past history of heart diseases and stroke, and medications for hypertension, diabetes, and dyslipidemia.

Model 4: Model 3 plus adjustment for diabetes, but excluding medications for hypertension, diabetes, and dyslipidemia.

Model 5: Model 3 plus adjustment for metabolic syndrome, but excluding medications for hypertension, diabetes, and dyslipidemia.

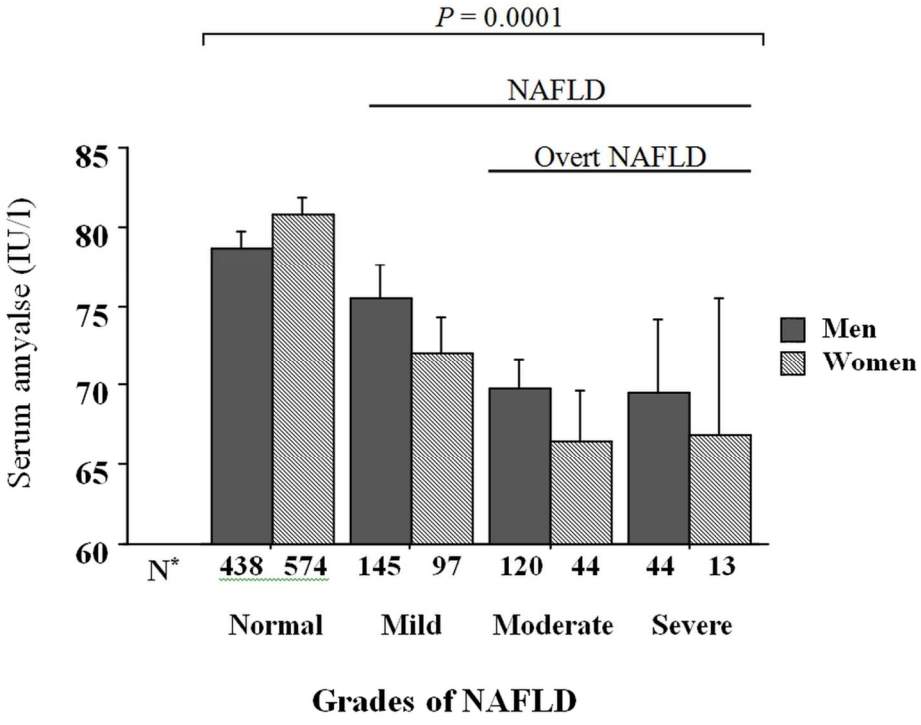
Model 6: Model 3 plus adjustment for BMI.

Model 7: Model 3 plus adjustment for WC.

Table 3. Odds ratios of each serum amylase for NAFLD of moderate or severe grade

Serum amylase quartiles	Q1 (Highest)	Q2	Q3	Q4 (Lowest)	*P* for trend
N*	320	316	308	289	
Model 1	1	1.29 (0.80–2.09)	2.04 (1.29–3.20)	3.51 (2.27–5.42)	< 0.0001
Model 2	1	1.36 (0.83–2.22)	2.14 (1.33–3.44)	3.81 (2.40–6.05)	< 0.0001
Model 3	1	1.61 (0.91–2.86)	2.32 (1.32–4.07)	3.45 (1.99–6.00)	< 0.0001
Model 4	1	1.48 (0.84–2.64)	2.11 (1.19–3.73)	2.97 (1.70–5.20)	< 0.0001
Model 5	1	1.56 (0.89–2.75)	2.32 (1.33–4.04)	3.26 (1.88–5.66)	< 0.0001
Model 6	1	1.05 (0.54–2.03)	1.44 (0.76–2.72)	2.01 (1.07–3.78)	0.01
Model 7	1	1.06 (0.55–2.05)	1.37 (0.73–2.59)	2.06 (1.09–3.87)	0.01

*Subjects with mild NAFLD (n = 242) were excluded from this analysis. Models 1–7 are the same as those in **Table 2**.



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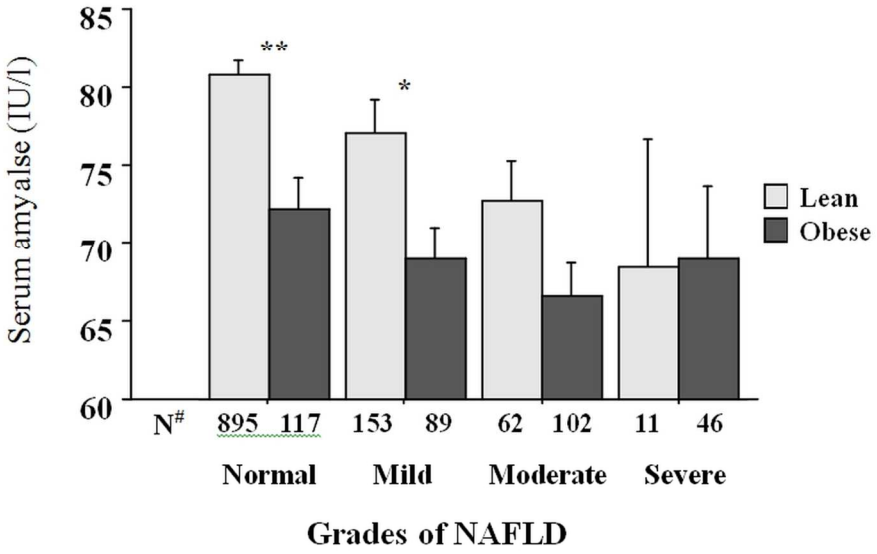

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Exclusion criteria of subjects and flow chart