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Afamin predicts the prevalence and incidence of nonalcoholic fatty liver disease

https://doi.org/10.1515/cclm-2021-0837
Received July 26, 2021; accepted November 15, 2021;
published online December 2, 2021

Abstract

Objectives: In the general population, increased afamin concentrations are associated with the prevalence and incidence of metabolic syndrome as well as type 2 diabetes. Although metabolic syndrome is commonly associated with nonalcoholic fatty liver disease (NAFLD), there exist no information on afamin and NAFLD.

Methods: Afamin concentrations were cross-sectionally measured in 146 Austrian patients with NAFLD, in 45 patients without NAFLD, and in 292 age- and sex-matched healthy controls. Furthermore, the feasibility of afamin to predict incident NAFLD was evaluated in 1,434 adult participants in the population-based Cardiovascular Risk in Young Finns Study during a 10-year follow-up.

Results: Median afamin concentrations were significantly higher in NAFLD patients (83.6 mg/L) than in patients without NAFLD (61.6 mg/L, p<0.0001) or in healthy controls (63.9 mg/L, p<0.0001). In age- and sex-adjusted logistic regression analyses a 10 mg/L increase of afamin was associated with a 1.5-fold increase of having NAFLD as compared with patients without NAFLD and the risk was even two-fold when compared with healthy controls. In the population-based cohort, afamin concentrations at baseline were significantly lower in participants without NAFLD (n=1,195) than in 239 participants who developed NAFLD (56.5 vs. 66.9 mg/L, p<0.0001) during the 10-year follow up, with highest afamin values observed in individuals developing severe forms of NAFLD. After adjustment for several potentially confounding parameters, afamin remained an independent predictor for the development of NAFLD (OR=1.37 [95% CI 1.23–1.54] per 10 mg/L increase, p<0.0001).

Conclusions: Afamin concentrations are increased in patients with NAFLD and independently predict the development of NAFLD in a population-based cohort.

Keywords: afamin; non-alcoholic liver disease; population-based studies; prediction; risk factors; vitamin E-binding protein.

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Introduction

Afamin is a highly glycosylated member of the albumin protein family, which in humans is encoded by genes located on chromosome 4q11–q13 [1]. It is predominantly expressed in the adult liver and secreted into the bloodstream; substantial amounts have also been found in follicular and cerebrospinal fluids [2]. Several associations between afamin and various disease phenotypes have been described. Afamin is a potential novel serum marker for ovarian cancer [3, 4], papillary thyroid carcinoma [5], cholangiocarcinoma [6] and increased oxidative stress in endometriosis [7]. In a previous pilot study, we observed decreased afamin concentrations in patients with heart failure, pneumonia, or sepsis [8]. The observed association between afamin and inflammatory biomarkers and disorders suggests that afamin could be an acute-phase protein, although the underlying pathophysiology remains to be clarified.

Previously, we conducted association studies in three large population-based cohorts in Germany, Italy and Austria and found highly significant positive associations between afamin plasma concentrations and prevalent and incident metabolic syndrome and each component thereof. These results were supported by a small pilot study in transgenic mice overexpressing human afamin [9, 10]. The association observed between afamin and metabolic syndrome in the general population was further confirmed in patients with polycystic ovary syndrome (PCOS), a condition with high prevalence of metabolic syndrome and insulin resistance [11, 12]. Afamin concentrations could be further shown to be significantly associated with prevalent and incident type 2 diabetes mellitus (T2DM) in a large multicenter population-based study of >20.000 individuals [13]. However, the mechanism underlying afamin's involvement in the development of metabolic syndrome and related diseases is currently unclear. A previously published review summarizes our current, limited knowledge on afamin's (patho)physiological functions [14].

Nonalcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease in developed countries, particularly during childhood [15], with an estimated global prevalence of up to 25% [16]. It is implicated in insulin resistance and dyslipidemia, and may be a link between afamin and components of the metabolic syndrome [17]. Kaikkonen et al. [18] have previously shown strong associations between a pattern of circulating lipids, lipoproteins, fatty acid and amino acid species and the incidence of NAFLD in the prospective Young Finns Study (YFS). Accordingly, we tested the hypothesis that increased afamin concentrations are associated with NAFLD and measured afamin serum/plasma concentrations in four different cohorts: NAFLD patients, patients without liver disease and healthy controls, as well as in a large population-based cohort with a 10-year follow-up.

Materials and methods

Cross-sectional prevalence study

Three study populations (Groups A, B and C) were formed from Austrian patient and control groups. The Innsbruck population included 191 patients who underwent examination at the Hepatology Outpatient Clinic of the Department of Internal Medicine II, Medical University of Innsbruck, Austria, between 2005 and 2012. Of these patients 146 (76%) had NAFLD as assessed by ultrasound and were designated the patient group with NAFLD (Group A). 10.3% of this group were treated with lipid-lowering drugs, 11.7% with antihypertensive and 6.2% with antidiabetic drugs. There were also 45 (24%) patients with irritable bowel syndrome, gastritis, fructose malabsorption, lactose intolerance, hypothyroidism or other diseases, but without NAFLD or other liver diseases; they were assigned to the non-NAFLD patient control group (Group B). 6.6% of this group received lipid-lowering medication, whereas 8.9% took antihypertensive drugs. Additionally, age- and sex-matched individuals were selected from the SAPHIR (Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk) study in a pairwise 2:1 design (using R-package "optmatch"). For this selection, participants with high alanine aminotransferase (ALAT) values were excluded (for men: ALAT>30 U/L, for women: ALAT>17 U/L), resulting in 292 healthy controls who were designated Group C. 3.8% of this selected subgroup from the SAHIR population received lipid-lowering drugs (statins, fibrates, or both), 14% took antihypertensive and 1.4% antidiabetic medication. The SAPHIR study is an observational study conducted from 1999 to 2002 involving 1,770 healthy unrelated Caucasian subjects (663 women (32.6%), 39–67 years of age and 1,107 men, 39–66 years of age, total mean 51 ± 6 years of age). Study participants were recruited through health screening programs in large companies in and around the city of Salzburg as described elsewhere [19].

Prospective incidence study

The Cardiovascular Risk in Young Finns Study (YFS), a population-based group of young Finnish adults was used for the longitudinal analysis. The YFS is an ongoing follow-up study of atherosclerotic precursors. The first cross-sectional survey was conducted in 1980 when 3,596 subjects aged 3–18 years participated [20]. Since then, follow-ups have been conducted in 1983, 1986, 2001, 2007 and 2011, when 2,063 participants (45% male, mean age 41.8 years in 2011) participated in clinical examinations. The present analyses are based on 1,509 participants with full data on afamin concentrations and clinical and laboratory determinants from the 2001 investigation. The incidence of NAFLD was studied in 1,434 participants in 2011 excluding 75 subjects with elevated ALAT activity suspected to have NAFLD at baseline in 2001. Exclusion criteria were the same as used in

the SAPHIR cohort (see above). NAFLD was assessed with ultrasound after a 10-year follow-up in 2011 and revealed a prevalence of 5% in normal-weight and 29% in obese subjects [21].

Due to the young age of this population, only 4.3% had received lipid-lowering and 11.0% antihypertensive medications, respectively, at the 10-years-follow-up investigation, as recently described [22]. At baseline (in 2001), only 0.3% reported taking lipid-lowering medications and only 2.5% antihypertensives.

All studies were *a priori* approved by the respective local human research committees and participants gave their written informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

In the YFS, baseline data in 2001 were acquired by self-administered questionnaires on alcohol consumption, which was transformed into standard drinks per day (corresponding to 12 g pure ethanol per standard drink). Body mass index (BMI) was determined as kg/m^2.

Participants were classified as having T2DM if, at any of the follow-up visits (2001, 2007 or 2011–2012), their fasting plasma glucose value was equal or greater than 7 mmol/L, or if they reported having a T2DM diagnosis made by a physician. In addition, individuals whose HbA_{1c} was equal or greater than 6.5% (48 mmol/mol) at 2011 follow-up or who reported taking glucose lowering medication at 2007 or 2011 follow-up were classified as having T2DM. Finally, T2DM diagnoses were obtained from the National Social Insurance Institution's Drug Reimbursement Registry.

Assessment of NAFLD

In groups A and B, NAFLD was assessed ultrasonically with a Hitachi EUB-7500A. In the YFS, ultrasound imaging of the liver was performed during the 2011 exam using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA, USA) with 4.0 MHz adult abdominal transducers. In all groups, ultrasound of the liver was performed using the validated NHANES III protocol [23]. NAFLD was visually graded in all patients by one trained ultrasonographer using liver-to-kidney contrast, parenchymal brightness, deep beam attenuation, bright vessel walls and visibility of the gall bladder neck [18, 24]. NAFLD subgroups were classified as normal (without fat accumulation detected by ultrasound), moderate (with mild fat accumulation) and severe NAFLD (with clear fat accumulation) based on sonographic assessment.

Biochemical analyses

Venous blood was drawn after overnight fasting and serum (in YFS and groups A and B) or EDTA plasma (group C) was obtained after low-speed centrifugation. Aliquots were stored at –70 °C until thawed for the first time for analyses.

Afamin was measured centrally at the Institute of Genetic Epidemiology of the Medical University of Innsbruck with photometric enzyme-linked immunosorbent assay (ELISA) on a semi-automated pipetting robot (Freedom Evo, Tecan Group, Männedorf, Switzerland) and ELISA reader (Benchmark Plus, Biorad Laboratories, Hercules, CA, USA). ELISA was a custom-made double-antibody sandwich assay (MicroCoat GmbH, Bernried, Germany) with a polyclonal rabbit antihuman-afamin antibody and the enzyme-conjugated monoclonal mouse anti-human afamin antibody N13. Within-run and total co-efficients of variation were 3.3 and 6.2%, respectively, at a mean concentration of 73 mg/L. No difference was observed between afamin measurements in serum and plasma samples [8].

In groups A and B, all routine laboratory parameters (liver enzymes, lipids, glucose, ferritin and CRP) were determined using routine laboratory methodology on a Roche Modular platform at the Central Institute of Medical and Chemical Diagnosis, University Hospital of Innsbruck, Austria.

All routine parameters in Group C were measured in the Central Laboratory of Christian-Doppler Clinic, Salzburg. Plasma fasting glucose and a complete lipid profile including fasting total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides were determined using routine laboratory procedures (Roche Diagnostics GmbH, Mannheim, Germany). CRP was measured with the Tina-quant Cardiac C-reactive Protein (Latex) High Sensitive kit from Roche Diagnostics, Mannheim, Germany, on a Hitachi 911 Chemistry Analyzer [25].

In the YFS, ASAT, ALAT and GGT activities were measured at the Department of Laboratory Medicine, Konventhospital Barmherzige Brueder Linz and Ordensklinikum Linz Barmherzige Schwestern, Linz, Austria with routine methods on an ARCHITECT automated analyzer (Abbott Diagnostics, Abbott Parks, IL, USA). All other biochemical analyses were performed at the Laboratory for Population Research of the National Institute for Health and Welfare (Turku, Finland). Serum concentrations of glucose, triglycerides, total cholesterol, HDL- and LDL cholesterol were determined using routine laboratory procedures [26, 27].

Statistical analyses

Descriptive characteristics of patients and controls and the YFS participants are presented as median (25th–75th percentile). Comparison of parameter distributions was performed with the Wilcoxon test between the patient cohort with NAFLD (group A) and each control group (groups B and C) as well as between participants with normal liver and with NAFLD in YFS.

Correlation between afamin and other continuous parameters was assessed using the Spearman correlation coefficient in the YFS as well as the groups A and B combined. Point bi-serial correlation was used to correlate afamin with sex.

Logistic regression models were performed to compare groups A and B. Conditional logistic regression was used in the models based on the age- and sex-matched healthy control group C. As no data concerning alcohol consumption was available for the Innsbruck patient cohort, alcohol consumption could not be accounted for in the corresponding regression models. Since the healthy control group C was selected using ALAT values and pairwise matched for age and sex, ALAT and sex were not taken as adjustment variables in the corresponding models.

The risk to develop NAFLD during the 10-year follow-up was studied in the YFS with a logistic regression model using subjects with normal liver function as controls and (both moderate and severe) NAFLD as cases.

In the Innsbruck patient and the YFS cohort different adjustment models were used: Model 1: adjusted for age and sex; Model 2: adjusted for age, sex, log-transformed ALAT, log-transformed triglycerides, log-transformed glucose, log-transformed CRP, T2DM and baseline BMI; Model 3 (only in the YFS): same as Model 2 with

additional adjustment for alcohol consumption and current BMI from 2011 instead of baseline BMI; Model 4 (only in the YFS): adjusted for age, sex and the presence of metabolic syndrome defined by the harmonizing criteria [28]; in Model 5 (only in the YFS), we additionally adjusted for the metabolites that predict fatty liver in the YFS population [18] (for details, see footnotes to Table 3).

All reported odds ratios refer to an increase in afamin per 10 mg/L after checking for linearity. Statistical analyses were performed using PASW Statistics 18.0 (IBM Inc., Armonk, NY, USA), R 3.0.1 in groups A, B and C, and R version 3.3.2 in the YFS.

Results

Cross-sectional study

Clinical and laboratory characteristics of NAFLD patients, patients without liver disease and healthy controls are shown in Table 1. NAFLD patients were significantly older, had a lower female/male ratio and significantly higher serum activities of ASAT, ALAT, GGT, and concentrations of glucose, triglycerides, CRP as well as lower concentrations of HDL cholesterol than did patients without NAFLD. Afamin concentrations were significantly higher in NAFLD patients than in patients without NAFLD (83.6 [72.7–100.0] mg/L vs. 61.6 [53.6–72.8] mg/L, p<0.0001, Table 1; Figure 1). Since these two patient groups showed major differences in age and sex distribution, we selected age- and sex-matched healthy controls from the SAPHIR study and observed similar differences in afamin concentrations between NAFLD patients and controls (83.6 [72.7–100.0] mg/L vs. 63.9 [55.6–72.2] mg/L, p<0.0001, Table 1; Figure 1).

In the combined patient groups, afamin correlated significantly and directly with BMI, ASAT, ALAT, GGT, glucose, total cholesterol, triglycerides, LDL cholesterol, and inversely with HDL cholesterol concentrations (Table 2), in agreement with and extending previous findings made in the general population [9].

Results for age-and sex-adjusted logistic regression analyses are shown in Table 3. An increase in afamin concentrations of 10 mg/L was associated with a 55% higher probability of having NAFLD as compared with patients without NAFLD (OR=1.55, p<0.0001); the risk was two-fold as compared with the healthy control group (OR=2.07, p<0.0001). After including several potentially confounding factors for adjustment, the association remained significant as compared with the patients without NAFLD (OR=1.35, p=0.0191) and remained basically unchanged as compared with the healthy control group (OR=2.07, p<0.0001).

Table 1: Clinical characteristics of groups A, B and C.

	Group A NAFLD patients (n=146)	Group B patients without liver disease (n=45)	Group C healthy controls (n=292)	p-Value comparing groups A with B	p-Value comparing groups A with C	p-Value comparing groups B with C
Females, n, %	22 (15%)	20 (44%)	44 (15%)	<0.0001	1	<0.0001
Age, years	53.6 (44.0–63.8)	39.5 (29.3–56.7)	54 (45–57)	0.0001	0.2098	<0.0001
BMI, kg/m^2	27.4 (24.8–30.1)	23.5 (20.4–25.9)	26.6 (24.0–29.1)	<0.0001	0.0249	<0.0001
Afamin, mg/L	83.6 (72.7–100.0)	61.6 (53.6–72.8)	63.9 (55.6–72.2)	<0.0001	<0.0001	0.7642
ALAT, U/L	52 (32–84)	24 (16–34)	14 (11–19)	<0.0001	<0.0001	<0.0001
ASAT, U/L	41 (31–57)	27 (23–32)	11 (9–13)	<0.0001	<0.0001	<0.0001
GGT, U/L	65 (39–193)	26 (19–69)	16 (11–24)	<0.0001	<0.0001	<0.0001
Glucose, mmol/L	5.49 (4.95–5.94)	5.27 (4.94–5.49)	5.05 (4.72–5.45)	0.0225	<0.0001	0.0555
Total cholesterol, mmol/L	5.34 (4.45–6.37)	4.94 (4.43–5.93)	5.79 (5.17–6.46)	0.2049	0.0002	0.0002
Triglycerides, mmol/L	1.97 (1.27–3.17)	1.15 (0.81–1.71)	1.12 (0.77–1.72)	<0.0001	<0.0001	0.7579
HDL cholesterol, mmol/L	1.27 (1.01–1.47)	1.40 (1.20–1.73)	1.47 (1.27–1.76)	0.0046	<0.0001	0.3771
LDL cholesterol, mmol/L	3.05 (2.43–3.86)	2.96 (2.48–3.59)	3.65 (3.04–4.33)	0.3858	<0.0001	<0.0001
CRP, mg/L	2.2 (1.2–4.2)	1.0 (0.6–2.5)	1.5 (0.7–2.8)	<0.0001	<0.0001	0.2280
Diabetes n, %	15 (10.3%)	1 (2.3%)	8 (2.7%)	0.1245	0.0022	1

Variables are expressed as median (25th–75th percentile); p-values are derived from the Wilcoxon test for continuous variables or the Chi-Square/Fisher's exact test for categorical variables.

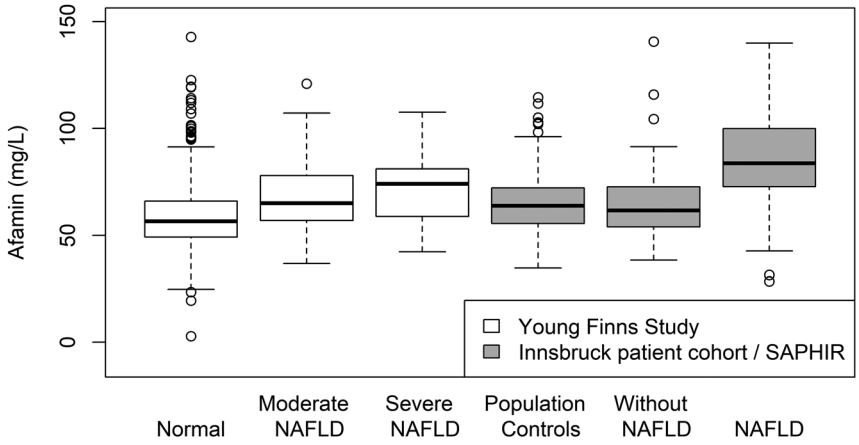

Figure 1: Afamin is associated with prevalent and incident NAFLD.
Boxplot showing afamin concentrations (mg/L) in the matched population control group (SAPHIR study, Group C, n=292), the Innsbruck patient cohort without NAFLD (Group B, n=45) and with NAFLD (Group A, n=146) and in 1434 YFS participants without (n=1,195), with moderate (n=195) and severe NAFLD (n=44).

Table 2: Correlation between afamin concentrations and anthropometric, laboratory and lifestyle parameters in groups A and B and the YFS.

	Groups A and B Combined (n=191)		YFS, excluding participants With elevated ALAT at baseline (n=1,434)	
	r	p-Value	r	p-Value
Sex	0.13	0.07	0.12	<0.0001
Age, years	0.06	0.43	−0.03	0.26
BMI, kg/m^2	0.25	0.0006	0.33	<0.0001
ASAT, U/L	0.29	<0.0001	0.20	<0.0001
ALAT, U/L	0.30	<0.0001	0.24	<0.0001
GGT, U/L	0.19	0.007	0.33	<0.0001
Glucose, mg/dL	0.15	0.04	0.14	<0.0001
Total cholesterol, mmol/L	0.19	0.01	0.29	<0.0001
Triglycerides, mmol/L	0.26	0.0003	0.44	<0.0001
HDL cholesterol, mmol/L	−0.17	0.02	−0.04	0.10
LDL cholesterol, mmol/L	0.19	0.01	0.20	<0.0001
CRP, mg/L	0.05	0.47	0.21	<0.0001
Alcohol consumption (standard drinks per day)	n/a	n/a	0.08	0.003

Prospective study

In a next step, we investigated whether afamin concentrations can predict the development of NAFLD in the long-term follow-up study of the YFS. The laboratory characteristics of this population without NAFLD at baseline are shown in Table 4.

Baseline median afamin concentrations were significantly higher in patients who developed NAFLD after a 10-year follow-up than in those without NAFLD (66.9 [57.6–78.2] mg/L vs. 56.5 [49.1–66.0] mg/L, p<0.0001). In Figure 1, afamin concentrations are shown separately in YFS participants without NAFLD (n=1,195), participants with moderate NAFLD (65.0 [56.9–78.0] mg/L, n=195) and participants with severe NAFLD (74.1 [59.3–81.0] mg/L, n=44, p<0.0001). Similar as in the Austrian patients, afamin correlated directly with BMI and with ASAT, ALAT, GGT, glucose, total and LDL cholesterol, triglycerides and CRP concentrations (Table 2).

In the YFS participants without elevated ALAT at baseline, afamin was a significant predictor of incident NAFLD in the 10-year follow-up (OR=1.02, p<0.006),

Table 3: Results of logistic or conditional logistic regression analysis.

Continuous effect of afamin (per 10 mg/L)		(Conditional) logistic regression		
	Model	**OR**	**95% CI**	**p-Value**
Cross-sectional study				
Groups A and B	Model 1	1.55	1.27–1.94	<0.0001
Cases: patients with NAFLD (Group A)	Model 2	1.35	1.06–1.77	0.0191
Controls: patients without NAFLD (Group B)				
Groups A and C	Model 1	2.07	1.72–2.49	<0.0001
Cases: patients with NAFLD (Group A)	Model 2	2.07	1.64–2.62	<0.0001
Controls: healthy sex- and age-matched (Group C)				
Prospective study				
YFS, excluding participants with elevated ALAT at baseline	Model 1	1.48	1.35–1.63	<0.0001
Cases: NAFLD	Model 2	1.18	1.05–1.32	0.005
Controls: normal liver	Model 3	1.14	1.01–1.25	0.032
	Model 4	1.44	1.30–1.60	<0.0001
	Model 5	1.37	1.23–1.54	<0.0001

Model 1: adjusted for age and sex (no adjustment for sex in the model containing groups A and C). Model 2: adjusted for age, sex, ALAT, triglycerides, glucose, CRP, type 2 diabetes (2011) and baseline BMI (no adjustment for sex and ALAT in the model containing groups A and C; n=1,422 in prospective study). Model 3 (only in YFS): adjusted for age, sex, ALAT, triglycerides, glucose, CRP, type 2 diabetes (2011), alcohol consumption and BMI from 2011 (n=1,419). Model 4 (only in YFS): adjusted for age, sex, and the presence of metabolic syndrome defined by the harmonizing criteria (≥3 of the following 5: waist ≥102 cm in men and ≥88 cm in women, fasting plasma glucose ≥5.6 mmol/L or treatment, hypertriglyceridemia ≥1.7 mmol/L and HDL cholesterol levels <1.0 mmol/L in men and <1.3 in women and blood pressure ≥130/≥85 mmHg or treatment) (n=1,386). Model 5 (only in YFS): adjusted for age, sex and metabolites as described [18] including VLDL particle concentration, VLDL triglycerides, serum saturated, monounsaturated and polyunsaturated fatty acids, as well as amino acids leucine and isoleucine (n=1,516).

Table 4: Baseline characteristics of subjects in the population-based YFS.

	NAFLD n=239	**Normal liver n=1,195**	**Comparing NAFLD with normal liver**
Females, n, %	74 (31)	743 (62)	<0.0001
Age, years	33.0 [30.0; 36.0]	33.0 [27.0; 36.0]	<0.001
BMI, kg/m^2	27.3 [24.8; 30.2]	23.7 [21.5; 26.0]	<0.001
Afamin, mg/L	66.9 [57.6; 78.2]	56.5 [49.1; 66.0]	<0.001
ALAT, U/L	11.0 [8.00; 17.0]	7.00 [6.00; 10.5]	<0.001
ASAT, U/L	19.0 [15.0; 24.0]	16.0 [13.0; 19.0]	<0.001
GGT, U/L	26.0 [20.0; 41.0]	16.0 [12.0; 23.0]	<0.001
Glucose, mmol/L	5.20 [4.90; 5.50]	4.90 [4.70; 5.20]	<0.001

Participants with elevated ALAT at baseline were excluded. Variables are expressed as median (25th–75th percentile); p-values were derived using Mann–Whitney test (continuous variables) or Fisher's exact test (categorical variables).

independently of ALAT, glucose, triglycerides, CRP, T2DM, age, sex, and baseline BMI (Model 2, Table 3). The result remained significant after further adjustment for alcohol consumption and current BMI (Model 3: OR=1.14, p=0.032), for the presence of metabolic syndrome (Model 4: OR=1.44, p<0.0001) and for the metabolic profile described by Kaikkonen et al. [18] (Model 5: OR=1.37, p<0.0001).

Discussion

Here we report a novel association between circulating concentrations of the vitamin E-binding protein afamin and prevalent as well as incident NAFLD. We applied two different study designs, a case-control design as well as a large prospective population-based design in two different ethniticies. The latter study population was most likely free of NAFLD at baseline based on an exclusion of individuals with elevated ALAT concentrations at baseline. Since NAFLD patients differed significantly from the relatively small group of patients without liver disease with respect to age and sex and in order to rule out potentially confounding factors in the control patient group, NAFLD patients were additionally compared with an age- and sex-matched healthy control group that was selected from a healthy working population. Significant differences in

afamin concentrations were observed between NAFLD patients and patients without NAFLD and the healthy control group.

The ability of afamin to predict the development of NAFLD was furthermore shown in the prospective follow-up in the YFS, where afamin was measured in serum samples collected in the 2001 examination and NAFLD was studied with ultrasound in 2011. A clear association between afamin concentrations and incident NAFLD was seen during the 10-year follow-up. Remarkably, the highest afamin values at baseline were seen in those individuals developing severe forms of NAFLD. Afamin predicted the development of NAFLD independently of known risk factors for NAFLD and of ALAT, which is an established marker of liver damage. This predictive power remained (although to a lesser extent) even after adjustment for established parameters for metabolic syndrome with which afamin was previously shown to be highly significantly associated [9]. Afamin remained significantly predictive for NAFLD even after adjustment for metabolic biomarkers as described by Kaikkonen et al. [18] demonstrating its independence from this particular metabolic profile. In addition, due to the relatively young age of the population and therefore low prevalence of disease outcomes and medication usage, it is very unlikely that such covariates would substantially influence the observed link between afamin and NAFLD.

This finding is, in our opinion, the key result of this study: while the literature reports no previous information on afamin concentrations in relation to NAFLD, we suggest that liver fat status may be the physiological factor linking afamin with components of metabolic syndrome and possibly affecting the bioavailability of afamin.

The biological roles of afamin have not been clarified. It has been suggested that afamin protects against oxidative stress by acting as carrier and transporter for α-tocopherol [29, 30], which has important anti-oxidative capacities. It has been hypothesized that the known binding affinity of serum albumin to heme is shared with other members of the paralogous albuminoids, including afamin [31]. Serum albumin has several known functions in the metabolism and transport of fatty acids, amino acids and metal ions, whereas α-fetoprotein is exclusively synthesized in fetal liver and yolk sac. Vitamin D-binding protein is known to bind to and transport vitamin D and its plasma metabolites.

Recently, we found afamin to be predictive for prevalent and incident metabolic syndrome and directly associated with the individual components of metabolic syndrome in three independent population-based cohorts [9]. Several studies have identified lowered circulating concentrations of afamin as being associated with malignancies [3, 5, 6] and oxidative stress [7]. We previously reported inverse associations between afamin and heart failure, pneumonia and sepsis [8]. A putative causative role of afamin in NAFLD might be explored utilizing known afamin genetic and genomic variants previously associated with NAFLD.

Interestingly, the PIVENS trial, a phase-3 multicenter clinical study for the treatment of NAFLD, revealed a significant improvement in non-alcoholic steatohepatitis after vitamin E therapy [32]. Since oxidative stress is considered a key contributor to the development of NAFLD (see review [33]), it is tempting to speculate that functional deficiency of vitamin E and other antioxidants may represent a potential pathogenic mechanism leading to NAFLD. Increased afamin concentrations in steatotic patients might therefore reflect the impaired balance between oxidative stress and available anti-oxidative defense in the human liver.

Our data are in line with a previously conducted proteomic study in children with NAFLD revealing afamin (among other candidates) as possible biomarker for NAFLD when compared with a control group without NAFLD [34]. These results were, however, not confirmed with a validated quantitative assay for afamin.

This study has several strengths and limitations: first, the afamin differences observed in the case-control study design are comparable between NAFLD patients on the one hand and patients without NAFLD and population-based healthy controls on the other hand, which supports the validity of our findings. Second, reported findings of an association between afamin and the prevalence of NAFLD in the Austrian cohorts are convincingly confirmed and extended by data on the incidence of NAFLD in the YFS. Lack of data regarding alcohol consumption in the Innsbruck cohorts has to be considered a limitation of the study, but one that is compensated by the observation that afamin's associations are independent of alcohol intake in the YFS. The relatively low number of non-NAFLD patients in the control group (B) has to be considered as further limitation, however, their data were confirmed by including the additional SAPHIR control group. Unfortunately, no data were available regarding oxidative stress markers or antioxidants both in the YFS and SAPHIR populations which could help explain a possible antioxidant role of afamin in the pathogenesis of NAFLD. NAFLD was semi-quantitatively assessed by liver ultrasound in our study which has limitations in terms of low sensitivity. The methodology was chosen for its feasibility to be applied in large population studies. Follow-up studies using alternative NAFLD diagnosis have therefore to be performed to supplement our

findings. Finally, the relative differences in afamin concentrations observed between the Finnish and Austrian cohorts might reflect ethnic differences; multicenter studies including different ethnic populations and appropriate controls are therefore needed.

Conclusions

We here describe a novel and predictive association between afamin concentrations and NAFLD. Afamin was already elevated long before NAFLD developed, namely when the participants with high baseline ALAT activities indicative of NAFLD were excluded from the longitudinal analyses. Therefore, afamin may serve as an early surrogate marker of events/diseases associated with NAFLD such as obesity, systemic hypertension, dyslipidemia, overt diabetes and – eventually – cardiovascular disease. Early diagnosis of NAFLD would thus help identify an increased risk for the development of metabolic syndrome and related diseases. Afamin as an early predictor of NAFLD could be used to assign risk patients to prevention programs and follow-up examinations.

Acknowledgments: The expert assistance in laboratory analysis, data collection and management, and statistical analyses, contributed by Linda Fineder, Nadja Baumgartner, Irina Lisinen, and Ville Aalto is gratefully acknowledged.
Research funding: The Young Finns Study has been financially supported by the Academy of Finland: Grants 322098, 286284, 134309 (Eye), 126925, 121584, 124282, 129378 (Salve), 117787 (Gendi), and 41071 (Skidi); the Social Insurance Institution of Finland; Competitive State Research Financing of the Expert Responsibility area of Kuopio, Tampere and Turku University Hospitals (Grant X51001); Juho Vainion Foundation; Paavo Nurmi Foundation; Finnish Foundation for Cardiovascular Research; Finnish Cultural Foundation; The Sigrid Juselius Foundation; Tampere Tuberculosis Foundation; Emil Aaltonen Foundation; Yrjö Jahnsson Foundation; Signe and Ane Gyllenberg Foundation; Diabetes Research Foundation of Finnish Diabetes Association. This project has received funding from the European Union's Horizon 2020 Research and Innovation Program under Grant Agreements No. 848146 for To Aition and Grant Agreement No. 755320 for TAXINOMISIS; European Research Council (Grant 742927 for MULTIEPIGEN project); Tampere University Hospital Supporting Foundation and Finnish Society of Clinical Chemistry. The Austrian contribution to this work has received support from grants of the Austrian Research Fund (P19969-B11) to H.D., Standortagentur Tirol and the Austrian Heart Fund to F.K., and Kamillo-Eisner Stiftung and Medizinische Forschungsgesellschaft Salzburg to B.P.
Conflict of interest statement: All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All studies were *a priori* approved by the respective local Human Research Committees.

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