Plasma Concentrations of Afamin Are Associated With the Prevalence and Development of Metabolic Syndrome

Florian Kronenberg, MD; Barbara Kollerits, PhD; Stefan Kiechl, MD; Claudia Lamina, PhD; Lyudmyla Kedenko, MD; Christa Meisinger, MD; Johann Willeit, MD; Cornelia Huth, PhD; Georg Wietzorrek, MD; Maria E. Altmann, PhD; Barbara Thorand, PhD; Andreas Melmer, MD; Doreen Dähnhardt, BS; Peter Santer, MD; Wolfgang Rathmann, MD; Bernhard Paulweber, MD; Wolfgang Koenig, MD; Annette Peters, MD, PhD; Ibrahim M. Adham, PhD; Hans Dieplinger, PhD

Background—Afamin is a human plasma vitamin E–binding glycoprotein primarily expressed in the liver and secreted into the bloodstream. Because little is known about (patho)-physiological functions of afamin, we decided to identify phenotypes associated with afamin by investigating transgenic mice overexpressing the human afamin gene and performing large-scale human epidemiological studies.

Methods and Results—Transgenic mice overexpressing afamin revealed increased body weight and serum concentrations of lipids and glucose. We applied a random-effects meta-analysis using age- and sex-adjusted baseline and follow-up investigations in the population-based Bruneck (n=826), Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR; n=1499), and KOoperative Gesundheitsforschung in der Region Augsburg (KORA) F4 studies (n=3060). Mean afamin concentrations were 62.5±15.3, 66.2±14.3, and 70.6±17.2 mg/L in Bruneck, SAPHIR, and KORA F4, respectively. Per 10 mg/L increment in afamin measured at baseline, the number of metabolic syndrome components increased by 19% (incidence rate ratio=1.19; 95% confidence interval [CI], 1.16–1.21; $P=5.62\times10^{-64}$). With the same afamin increment used at baseline, we observed an 8% gain in metabolic syndrome components between baseline and follow-up (incidence rate ratio=1.08; 95% CI, 1.06–1.10; $P=8.87\times10^{-16}$). Afamin concentrations at baseline were highly significantly related to all individual metabolic syndrome components at baseline and at follow-up. This observation was most pronounced for elevated waist circumference (odds ratio, 1.79; 95% CI, 1.54–2.09; $P=4.15\times10^{-14}$ at baseline and odds ratio, 1.46; 95% CI, 1.31–1.63; $P=2.84\times10^{-11}$ for change during follow-up) and for elevated fasting glucose concentrations (odds ratio, 1.46; 95% CI, 1.40–1.52; $P=1.87\times10^{-69}$ and odds ratio, 1.46; 95% CI, 1.24–1.71; $P=5.13\times10^{-6}$, respectively).

Conclusions—This study in transgenic mice and >5000 participants in epidemiological studies shows that afamin is strongly associated with the prevalence and development of metabolic syndrome and all its components. **(*Circ Cardiovasc Genet*. 2014;7:822-829.)**

Key Words: afamin protein, human ■ epidemiology ■ glucose ■ lipoproteins ■ metabolism ■ metabolic syndrome X ■ obesity

Afamin was discovered in 1994 as the fourth member of the human albumin gene family, which includes human serum albumin, alfa-fetoprotein, and vitamin D–binding protein.[1] All 4 genes map to the chromosomal region 4q11-q22. Afamin is also known as α-albumin or as α1T-glycoprotein.[2,3] Afamin is a human plasma glycoprotein of 87 kDa with 15% carbohydrate content and 55% amino acid sequence similarity to albumin. Circulating plasma afamin is primarily of hepatic origin[1]; brain, kidney, testes, and ovaries have been found as additional afamin-expressing tissues (www.proteinatlas.org). Abundant concentrations of afamin have been described in plasma and in other body fluids, such as follicular, cerebrospinal and seminal

Clinical Perspective on p 829

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From the Division of Genetic Epidemiology (F.K., B.K., C.L., D.D., H.D.), Division of Molecular and Cellular Pharmacology, Department of Medical Genetics, Molecular, and Clinical Pharmacology (G.W.), Department of Neurology (S.K., J.W.), Department of Internal Medicine I (A.M.), Innsbruck Medical University, Innsbruck, Austria; First Department of Internal Medicine, Paracelsus Private Medical University, Salzburg, Austria (L.K., B.P.); Institute of Epidemiology II, Helmholtz Zentrum München—German Research Center for Environmental Health, Oberschleißheim, Germany (C.M., C.H., B.T., A.P.); German Center for Diabetes Research (DZD), Neuherberg, Germany (C.M., C.H., B.T., A.P.); Institute of Human Genetics, University of Göttingen, Göttingen, Germany (M.E.A. I.M.A.); Department of Laboratory Medicine, Bruneck Hospital, Bruneck, Italy (P.S.); Institute of Biometrics and Epidemiology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine, University Düsseldorf, Düsseldorf, Germany (W.R.); Department of Internal Medicine II—Cardiology, University of Ulm Medical Center, Ulm, Germany (W.K.); and Vitateq Biotechnology GmbH, Innsbruck, Austria (H.D.).

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Correspondence to Hans Dieplinger, PhD, Division of Genetic Epidemiology, Department of Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck Medical University, Schöpfstrasse 41, A-6020 Innsbruck, Austria. E-mail hans.dieplinger@i-med.ac.at

fluid. Strong correlations between afamin concentrations in these fluids and in plasma suggest a hepatic origin of afamin also in these extravascular compartments.[4]

Little is known about the physiological or pathophysiological functions of afamin. We previously demonstrated multiple vitamin E–binding sites of human afamin using a radioligand assay. Scatchard and Hill analyses showed binding affinity for both α- and γ-tocopherol.[5]

To deepen our understanding of possible functions of afamin, we generated transgenic mice overexpressing the human afamin gene. Plasma concentrations of lipids and lipoproteins (non–high-density lipoprotein [HDL] cholesterol and triglycerides), as well as the mean body weight of transgenic animals were significantly elevated when compared with those of age- and sex-matched wild-type littermates. These findings of a hyperlipidemic, hyperglycemic, and obese phenotype suggest a possible role of afamin in frequently observed metabolic disturbances, such as metabolic syndrome. To investigate a possible human correlation to the previously observed findings in the mouse model, we initiated large-scale measurements of afamin plasma concentrations in 2 population-based cohorts from Italy and Germany and 1 cohort of a healthy working population from Austria. Therefore, our primary hypothesis was that afamin is associated with metabolic syndrome in these populations, either in a cross-sectional manner or prospectively. In a further step, we evaluated whether the association with metabolic syndrome is triggered by one of its single components.

Methods

Generation and Phenotyping of Afamin-Transgenic Mice

FVB/N mice[6] overexpressing human afamin were created using a vector carrying human afamin cDNA under the control of enhancer and promoter regions of the murine albumin gene. The 3′untranslated region of human growth hormone gene was inserted into the 3′end of afamin cDNA.[7] The construct was microinjected into male pronuclei of fertilized eggs, which were then transferred into pseudopregnant mice. Six homozygous transgenic lines with copy numbers between 3 and 180 were obtained. Mouse line PH27 (n=8) with the highest transgene copy number (180) was used for phenotype analyses and compared with wild-type FVB/N mice (n=8). Blood from 12-month-old male animals was obtained after 18-hour fasting by retro-orbital puncture and serum generated by low-speed centrifugation. Serum concentrations of triglycerides, total and HDL cholesterol, and glucose were measured using routine colorimetric assays (Reflotron; Roche, Darmstadt, Germany), and expression levels of afamin were determined with ELISA using antibodies raised against human afamin (see below).

Human Study Populations, Design, and Assessments

Subjects enrolled in 3 independent white cohorts recruited from the general population in the Bruneck Study and the Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR) study and from a healthy working population in the KOoperative Gesundheitsforschung in der Region Augsburg (KORA) F4 study served as the basis for the present epidemiological investigations. All clinical investigations were conducted according to the Declaration of Helsinki. Participants from all 3 studies provided written informed consent, and the studies were approved by the local ethical committees of Bolzano and Verona (Bruneck study), Land Salzburg (SAPHIR study), and the Bayerische Landesärztekammer (KORA F4 study).

The Bruneck study is a prospective, population-based study without specific exclusion criteria that aims to investigate the epidemiology and pathogenesis of atherosclerosis and related traits.[8,9] At study baseline in 1990, a random sample including 1000 subjects recruited from the entire population of Bruneck stratified according to sex and age with 125 subjects of each sex for each decade of age between 40 and 79 (mean, 58±11) years were invited to participate. The participation rate was 93.6% with complete data in 919 subjects. All participants were of white origin. Follow-up examinations were performed every 5 years. The 1995 and 2000 examinations were the basis for the present data analysis including 826 subjects of whom all had afamin data available. Demographic data, medications, and clinical history, as well as atherosclerosis risk profile including smoking and drinking behavior, were recorded by questionnaire, as well as standardized interview. Detailed information on prevalent and incident metabolic syndrome components was available from all examinations. EDTA blood samples were taken after fasting and abstaining from smoking for ≥12 hours. After centrifugation, plasma was stored at −70°C.[8] All laboratory measurements were performed in samples collected in 1995.

The SAPHIR study is an observational study conducted from 1999 to 2002 involving 1770 healthy unrelated white subjects (663 women [32.6%]; age, 39–67 years and 1107 men; age, 39–66 years; total mean age, 51±6 years). Afamin data were available from 1499 subjects. Study participants were recruited through health screening programs in large companies in and around the city of Salzburg as described.[10] Only individuals without diseases or conditions that might have biased or influenced clinical outcomes related to atherosclerotic diseases were recruited and subjects with established coronary artery, cerebrovascular or peripheral arterial disease, congestive heart failure, valvular heart disease, chronic alcohol intake of ≥3 drinks a day, drug abuse, or morbid obesity (body mass index [BMI], >40 kg/m^2) and pregnant women were excluded.[10] Follow-up examinations including 1388 subjects were conducted between 2002 and 2008 corresponding to a mean follow-up time of 4.57 years; afamin data at baseline were available from 1171 of them. Physical examination included measurement of anthropometric parameters, such as weight, height, and waist circumference. At the baseline investigation, there were 18 individuals who met metabolic syndrome criteria only because of hypertensive medication, 2 because of diabetes mellitus medication, and 2 because of fibrate medication. At the follow-up investigation, there were 59 individuals who met metabolic syndrome criteria only because of hypertensive medication, 1 because of diabetes mellitus medication, and 17 because of fibrate medication. These numbers are based on the individuals with afamin data available (n=1499 at baseline and n=1171 at follow-up). Venous EDTA blood was collected after overnight fasting; plasma was obtained by low-speed centrifugation and stored at −70°C until analyses.

The Cooperative Health Research in the Region of Augsburg (KORA) study incorporates representative cohorts of the general white population in Augsburg, Germany, and 2 surrounding counties and was initiated as part of the World Health Organization MONItoring of trends and determinants in CArdiovascular diseases (WHO MONICA) study. The KORA S4 survey included 4261 men and women aged between 25 and 74 years, with a response rate of 67% and without any specific exclusion criteria. Seven years later in 2006/2008, KORA F4 was performed with a total of 3080 participants as the follow-up study to KORA S4. The present investigation included 3060 subjects from the KORA F4 study for whom data on afamin were available. In KORA F4, there was no further follow-up examination. Standardized face-to-face interviews were performed by certified medical staff and standardized medical examinations including blood analyses (collected after overnight fasting for ≥10 hours), and anthropometric measurements were conducted in all participants.[11]

Definition of Metabolic Syndrome

Metabolic syndrome was defined according to the joint statement of the American Heart Association and the National Heart, Lung, and Blood Institute.[12] Three of the following 5 parameters had to be present: fasting triglycerides ≥150 mg/dL or on drug treatment for elevated triglycerides (fibrates and nicotinic acids); HDL cholesterol <40 mg/dL in men, <50 mg/dL in women or on drug treatment for reduced HDL cholesterol (fibrates and nicotinic acids); fasting glucose ≥100 mg/dL or on drug treatment for elevated glucose; hypertension defined as systolic blood pressure ≥130 mm Hg or diastolic blood pressure ≥85 mm Hg or

antihypertensive drug treatment in a patient with a history of hypertension; waist circumference ≥102 cm in men, ≥88 cm in women. For supplementary sensitivity analysis, we applied the definition of metabolic syndrome published by the International Diabetes Foundation.[13]

Other Clinical Parameters

In all 3 studies, hypertension was defined as described above. Participants were diagnosed as having type 2 diabetes mellitus if their fasting plasma glucose concentration was ≥7 mmol/L and they were being treated with antidiabetic therapy (medication and diet). BMI was calculated as weight (kg) divided by height (m) squared. Obesity was defined as a BMI≥30 kg/m^2.

Measurements of Afamin Plasma Concentrations and Other Laboratory Values

Afamin was quantified as previously described[4,5] with a custom-made double-antibody sandwich ELISA using an affinity-purified biotinylated polyclonal antiafamin antibody for coating 96-well streptavidin-bound microtiter plates and peroxidase-conjugated monoclonal antibody N13 for detection (MicroCoat Biotechnologie GmbH, Bernried, Germany). Secondary plasma in serial dilutions initially calibrated with a primary standard served as the assay standard. Afamin purified to homogeneity from human plasma was used as primary standard; its exact protein concentration was determined by quantitative amino acid compositional analysis. Within-run and total coefficients of variation were 3.3% and 6.2%, respectively, at a mean concentration of 73 mg/L.[14] All afamin concentrations were measured in our laboratory using the same method. Lipid and liver parameters were determined by routine commercial assays (Data Supplement).

Statistical Analyses

Distributions of all relevant parameters were compared between afamin-transgenic mice and wild-type controls by means of Wilcoxon rank-sum tests.

To compare baseline characteristics in human studies, subjects from all 3 study populations were stratified into tertiles of baseline afamin concentrations. Tertile groups of afamin were calculated based on pooled data from the Bruneck, SAPHIR, and KORA studies. One-way ANOVA or the Kruskal–Wallis test was applied for continuous variables, where appropriate. Dichotomized variables were compared using Pearson χ^2 test. Non-normally distributed variables were transformed based on the natural logarithm for further analyses. Correlations between afamin and all continuous metabolic syndrome components were calculated using Spearman rank correlation coefficient. Correlations between binary variables (sex and diabetes mellitus) and afamin were assessed using the point-biserial correlation coefficient and between smoking and afamin with Kendall τ correlation coefficient. In addition, correlation plots for afamin with continuous metabolic syndrome components are provided. At baseline, unadjusted mean values and 95% confidence intervals (CIs) of plasma afamin concentrations per metabolic syndrome component are displayed. Furthermore, marginal mean values and 95% CI of baseline plasma afamin concentrations (adjusted for the number of metabolic syndrome components at baseline) are presented per change in number of metabolic syndrome components between baseline and follow-up. To explore the association between afamin and metabolic syndrome, a logistic regression analysis of the presence of metabolic syndrome was applied. The number of components was modeled using a Poisson regression model assuming count data as the outcome variable. To account for possible overdispersion, which means that the mean and variance of the underlying Poisson distribution are not equal, a quasi-likelihood was applied (quasi-Poisson regression: using function glm in R). Using this model, an incidence rate ratio was estimated reflecting the percentage increase in number of components. To evaluate whether the association with metabolic syndrome is triggered by 1 single component, additional logistic regression analyses were applied for each single metabolic syndrome component present. In addition, linear regression models were calculated on the continuous component variables. Because HDL cholesterol and waist circumference differ between men and women, regression models on these traits were calculated separately for men and women, and each analysis was adjusted for age. All remaining analyses were adjusted for age and sex. Furthermore, all linear regression models at baseline were adjusted for medication at baseline, where appropriate. Because the individual components are part of the metabolic syndrome definition and thus not independent of it, no correction for multiple testing was applied for these additional secondary analyses.

Using the baseline afamin values, we also conducted analyses of metabolic syndrome components at the follow-up time point, additionally adjusting for the number of components at baseline. We also applied linear and logistic regression models for the individual components at follow-up. The linear regression analyses were also adjusted for medication at baseline, where appropriate. As a sensitivity analysis, we applied a logistic regression model at follow-up with the metabolic syndrome yes/no variable as the outcome only in those individuals free of metabolic syndrome at the baseline investigation. We tested the linearity of the continuous covariates (ie, afamin and age) by applying a penalized regression spline approach. There was no indication for nonlinearity of afamin or age effects in any of the applied logistic regression models at baseline or follow-up.

A pooled effect size for the respective studies was calculated via random-effects meta-analysis. A 2-sided P value <0.05 was considered statistically significant. Analyses were performed using SPSS for Windows, version 20.0, and R 2.14.2.

Results

Afamin-Transgenic Mice

Adolescent (6–8 weeks old) transgenic animals reproduced normally, and we observed no grossly obvious phenotype differing from that of wild-type mice of the same genetic FVB/N background at that age. One-year-old male and female animals, however, had a 20% higher body weight than did age-matched wild-type littermates (Figure 1). Mean serum concentrations of human afamin in transgenic mice reached 24.3 mg/L, equivalent to ≈40% of human afamin plasma values[4] and were ≈10× higher than those of wild-type mice, the latter most likely being the result of antibodies cross-reacting with endogenous mouse

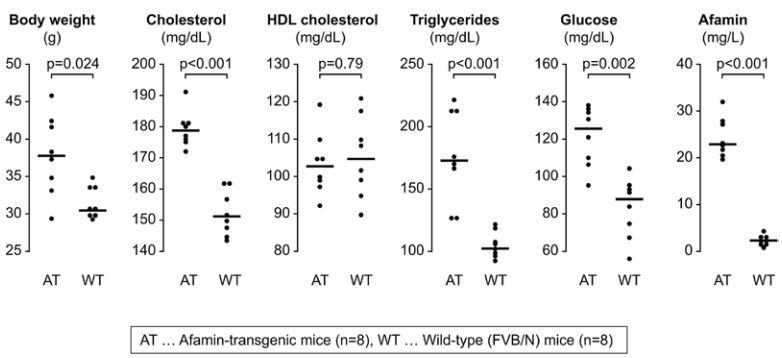


Figure 1. Basic phenotypes in 1-year-old fasting afamin-transgenic (AT) and wild-type (WT) mice of the same FVB/N genetic background. Horizontal bars represent medians; P values of the Wilcoxon test are given. HDL indicates high-density lipoprotein.

afamin. Most remarkably, concentrations of total cholesterol, triglycerides, and glucose were significantly increased by 17%, 69%, and 45%, respectively, whereas HDL cholesterol was not different than in sex-matched wild-type littermates (Figure 1).

Association Between Afamin and Metabolic Syndrome in Humans at Baseline

Encouraged by the findings in transgenic afamin animals, we investigated the association between afamin and various metabolic parameters along with basic anthropometrics in 3 large general population cohorts. In these 3 cohorts, mean afamin concentrations were 62.5±15.3 (Bruneck), 66.2±14.3 (SAPHIR), and 70.6±17.2 mg/L (KORA F4; Table 1). Afamin concentrations met the specifications that define a normal distribution as demonstrated in the largest study, KORA F4, in Figure I in the Data Supplement. There were slight but significant correlations between afamin and age in the SAPHIR and KORA studies and sex in the KORA study. Study participants from each population were stratified into tertiles according to afamin plasma concentrations (Tables IA–C in the Data Supplement). For each of the 3 populations, we observed pronounced and positive associations between afamin plasma concentrations and mean values of waist circumference, BMI, systolic and diastolic blood pressure, total and low-density lipoprotein cholesterol, triglycerides, and glucose, as well as prevalent diabetes mellitus and obesity. Afamin plasma concentrations showed an inverse association with mean HDL cholesterol and a slight, but significant positive association with median high-sensitivity C-reactive protein (CRP) concentrations (Tables IA–C in the Data Supplement). In the SAPHIR study, we compared those participants with and without afamin data (Table II in the Data Supplement). The strongest univariate correlations were observed between afamin and the metabolic syndrome component parameters triglycerides and waist circumference (Table III; Figure I in the Data Supplement).

As shown in Figure 2, mean afamin plasma concentrations correlated positively with the number of metabolic syndrome components at baseline in all 3 investigated study populations. This increase was shown to be highly significant in a quasi-Poisson model (primary analysis): per 10 mg/L increment in afamin measured at baseline, the number of components increased by 19% (incidence rate ratio=1.19; 95% CI, 1.16–1.21; $P=5.62\times10^{-64}$; Table 2). The logistic regression analysis (primary analysis) showed a 79% increased probability of being diagnosed with metabolic syndrome (when ≥3 components are present) per 10 mg/L increase in afamin (OR, 1.79; 95% CI, 1.63–1.96; $P=5.02\times10^{-35}$; Table 2). In the secondary analysis, afamin plasma concentrations were highly significantly related to all individual metabolic syndrome components at baseline in all 3 studies, irrespective of whether the continuous variable components (Table V in the Data Supplement) or the defined and accepted cutoff for the individual components were used (Table 2). This association was most pronounced for an elevated waist circumference (OR=1.79). Even after additionally adjusting for smoking status and ln CRP (as performed in Table 2 for age and sex), effect estimates did not change (Table IVA in the Data Supplement).

Table 1. Distribution of Demographic and Laboratory Parameters, Medication, Afamin, and Metabolic Syndrome in the Bruneck, SAPHIR, and KORA F4 Study Groups

	Bruneck (n=826)	SAPHIR (n=1499)	KORA (n=3060)
Age, y	57.9±11.1	51.0±6.0	56.1±13.3
Sex (men/women), %	50.1/49.9	67.4/32.6	48.3/51.7
Afamin, mg/L	62.5±15.3	66.2±14.3	70.6±17.2
Metabolic syndrome, %	32.6	24.3	30.1
Hypertension, %	85.0	76.0	50.8
Elevated waist circumference. %	29.2	38.6	44.6
Reduced HDL cholesterol, %	18.6	10.5	20.4
Elevated triglycerides, %	29.2	25.3	25.4
Elevated glucose, %	41.1	20.3	33.2
Medication used for metabolic syndrome definition			
Fibrate use, %	1.2	0.2	0.5
Diabetes medication, %	4.6	1.5	5.9
Blood pressure medication, %	27.6	14.2	30.9

Data are presented as mean±SD or (%) if not indicated otherwise. Extended characteristics are provided in the Data Supplement. HDL indicates high-density lipoprotein; KORA, KOoperative Gesundheitsforschung in der Region Augsburg; and SAPHIR, Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk.

Association Between Afamin at Baseline and Metabolic Syndrome at Follow-Up

To investigate a possible predictive role of afamin plasma concentrations in the development of metabolic syndrome, we

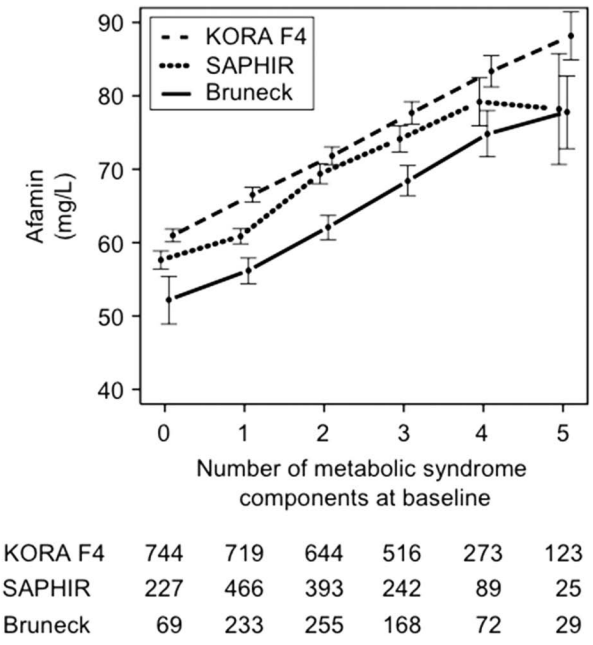


Figure 2. Association between unadjusted mean plasma afamin concentrations (95% confidence interval) and number of metabolic syndrome components at baseline in 3 independent human populations: Bruneck study (n=826), Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR) study (n=1499), and KOoperative Gesundheitsforschung in der Region Augsburg (KORA F4) study (n=3060). n represents the number of participants with the respective number of metabolic syndrome components in each of the studies.

Table 2. Poisson and Logistic Regression Analyses of Afamin for Metabolic Syndrome and Its Components at Baseline (Adjusted for Age and Sex)

	Bruneck		SAPHIR		KORA F4		Meta-analysis*	
	IRR/OR (95% CI)†	*P* Value	IRR/OR (95% CI)†	*P* Value	IRR/OR (95% CI)†	*P* Value	IRR/OR (95% CI)†	*P* Value
Primary analyses								
Quasi-Poisson regression								
No. of metabolic syndrome components	1.18 (1.16–1.21)	2.22×10^{-16}	1.21 (1.19–1.24)	1.58×10^{-67}	1.17 (1.16–1.19)	2.58×10^{-114}	1.19 (1.16–1.21)	5.62×10^{-64}
Logistic regression for metabolic syndrome								
Metabolic syndrome AHA/NHLBI (yes/no)	1.92 (1.69–2.17)	5.09×10^{-25}	1.86 (1.69–2.05)	1.65×10^{-36}	1.67 (1.57–1.77)	2.10×10^{-67}	1.79 (1.63–1.96)	5.02×10^{-35}
Metabolic syndrome IDF (yes/no)	1.77 (1.57–1.98)	4.56×10^{-22}	1.80 (1.64–1.98)	7.72×10^{-35}	1.64 (1.55–1.73)	8.19×10^{-65}	1.71 (1.60–1.83)	2.11×10^{-56}
Secondary analyses								
Logistic regression for components of the metabolic syndrome								
Hypertension (yes/no)	1.45 (1.26–1.69)	6.31×10^{-7}	1.44 (1.30–1.60)	1.64×10^{-12}	1.27 (1.20–1.34)	6.00×10^{-19}	1.37 (1.24–1.51)	1.23×10^{-09}
Elevated waist circumference (yes/no)	1.73 (1.53–1.96)	4.15×10^{-18}	2.07 (1.87–2.29)	7.81×10^{-45}	1.63 (1.54–1.72)	1.39×10^{-66}	1.79 (1.54–2.09)	4.15×10^{-14}
Reduced HDL (yes/no)	1.35 (1.20–1.52)	7.99×10^{-7}	1.25 (1.12–1.40)	5.32×10^{-5}	1.21 (1.15–1.27)	6.40×10^{-13}	1.25 (1.17–1.33)	2.75×10^{-12}
Elevated fasting triglycerides (yes/no)	1.92 (1.69–2.18)	1.14×10^{-24}	1.64 (1.50–1.79)	9.61×10^{-27}	1.60 (1.51–1.69)	4.04×10^{-60}	1.69 (1.54–1.85)	4.59×10^{-28}
Elevated fasting glucose (yes/no)	1.41 (1.27–1.56)	4.54×10^{-11}	1.52 (1.38–1.66)	3.63×10^{-19}	1.45 (1.38–1.53)	1.82×10^{-42}	1.46 (1.40–1.52)	1.87×10^{-69}

AHA indicates American Heart Association; CI, confidence interval; HDL, high-density lipoprotein; IDF, International Diabetes Foundation; IRR, incidence rate ratio; KORA, KOoperative Gesundheitsforschung in der Region Augsburg; NHLBI, National Heart, Lung, and Blood Institute; OR, odds ratio; and SAPHIR, Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk.

*Meta-analysis results based on a random-effects model.

†Afamin increment is 10 mg/L.

correlated baseline afamin values to the change in number of metabolic syndrome components after a follow-up period of ≈5 years when compared with the baseline investigation in the Bruneck and SAPHIR study populations. There was no further follow-up investigation for KORA F4. Figure 3 shows a positive association between mean afamin baseline values and the change in the number of these components, adjusted for the number of components at baseline, indicating that afamin is predictive for developing metabolic syndrome. In both studies, the corresponding quasi-Poisson model (Table 3, primary analysis) showed a significant association with the number of components at follow-up (incidence rate ratio combined 1.08; 95% CI, 1.06–1.10; $P=8.87\times10^{-16}$). With the same afamin increment as used at baseline an 8% gain in metabolic syndrome components between baseline and follow-up was observed. Highly significant associations could also be found in all logistic regression analyses (Table 3), whether for the presence of metabolic syndrome (primary analysis) or for all individual components at follow-up (secondary analysis). Similarly, as shown for the associations at baseline, additional adjusting for smoking status and ln CRP did not change the effect estimates (Table IVB in the Data Supplement). The associations based on the metabolic syndrome components defined by the respective cutoffs were more pronounced than were those based on the continuous components (Table VI in the Data Supplement). Baseline afamin's strongest association was seen with elevated waist circumference and fasting glucose concentrations at follow-up (both OR=1.46).

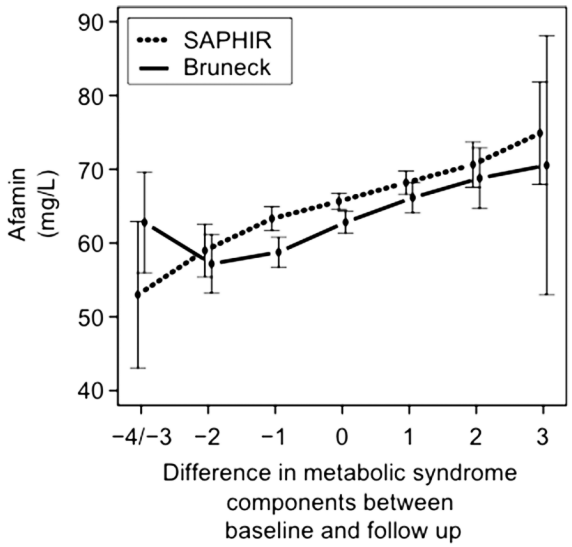
Sensitivity Analyses

When we performed a sensitivity analysis in only those individuals free of metabolic syndrome at the baseline investigation, we observed per 10-mg/L increase in afamin an 82% higher risk for developing a metabolic syndrome at follow-up (OR, 1.82; 95% CI, 1.60–2.07; $P=7.99\times10^{-20}$) in the SAPHIR and Bruneck cohorts combined. When additionally excluding those with prevalent and incident diabetes mellitus, estimates pointed in the same direction in the combined analysis (OR, 1.81; 95% CI, 1.59–2.07), $P=8.47\times10^{-19}$; Table 3).

In a further sensitivity analysis, we applied the International Diabetes Foundation criteria for the definition of metabolic syndrome. As shown in Tables 2 and 3, estimates of the meta-analyses at baseline and follow-up did not change when compared with American Heart Association and the National Heart, Lung, and Blood Institute (OR, 1.71; 95% CI, 1.60–1.83; $P=2.11\times10^{-56}$ and OR, 1.57; 95% CI, 1.42–1.73; $P=3.73\times10^{-19}$, respectively).

Discussion

On the basis of results from a study in transgenic mice overexpressing human afamin, we investigated a possible association between afamin and components of metabolic syndrome in 3 independent, human population-based white cohorts. Afamin plasma concentrations were found to be consistently associated with both the prevalence (in all 3 populations) and the incidence of metabolic syndrome, which was investigated only in the Bruneck and SAPHIR studies. Afamin plasma



n SAPHIR	6	47	222	511	236	62	12
n Bruneck	14	41	151	289	148	37	2

Figure 3. Association between mean plasma afamin concentrations (95% confidence interval) measured at baseline and the change in number of metabolic syndrome components between baseline and follow-up investigation 5 years later in the Bruneck and Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR) study populations. Data were adjusted for metabolic syndrome components at baseline. n provides the number of participants with the respective change in number of metabolic syndrome components during the observation period for each of the 2 studies. Because of the small number, we combined the stratum with a decrease in 3 and 4 metabolic syndrome components into 1 category.

concentrations were also significantly associated with the individual anthropometric and metabolic risk factors relevant for the development of metabolic syndrome. In particular, the association between afamin and metabolic syndrome was not only ascribed to 1 specific metabolic syndrome component but also found for all contributing components. These findings are essentially in line with and extend the associations between afamin plasma concentrations and features of metabolic disorders in the afamin-transgenic mouse model.

The only discrepancy between the epidemiological human data and those from the transgenic mouse model was observed regarding HDL cholesterol. Although afamin was strongly associated with HDL cholesterol concentrations in all 3 human populations, no difference was seen in HDL between transgenic mice and wild-type animals. Afamin overexpression obviously affected only apoB-containing lipoproteins without significantly changing HDL, possibly because of lacking cholesterol ester transfer protein being responsible for exchanging cholesterol esters and triglycerides between HDL and apoB-containing lipoproteins.[15]

The search for key factors causing metabolic syndrome besides fat- and carbohydrate-rich overnutrition and a sedentary lifestyle was until now disappointing. One of the driving forces might indeed be abdominal obesity that is an estimated origin for systemic inflammation, which in turn correlates with both the development and the incidence of insulin resistance and type 2 diabetes mellitus.[16] Visceral adipose tissue was identified as an important source of interleukin-6, tumor necrosis factor-α, and plasminogen activator inhibitor 1 triggering systemic inflammation.[17–22] Therefore, it is interesting that afamin showed strong associations, particularly with waist circumference. The mechanistic and functional insight into afamin's involvement in the development of metabolic syndrome or the pathogenesis of individual metabolic syndrome components are currently unclear. Experimental studies in human and murine cell models, as well as animal studies, will have to investigate the influence of afamin on components of insulin resistance syndrome. These experiments will also elucidate the regulation of afamin expression. Furthermore, it will be of utmost importance to identify ligands and interaction partners of afamin to elucidate structural–functional properties of afamin.

About the mechanisms and possible causality of our findings, it is tempting to speculate why the afamin gene (*AFM*) has not been previously identified in genome-wide association studies of metabolic syndrome, diabetes mellitus, glucose, BMI, waist, or other metabolic syndrome-related variables. Although afamin is strongly correlated with metabolic syndrome and its components, genetic variance likely explains only a small part of the variability of these components. It is not yet known how much of the afamin concentration is genetically regulated, either at a general level or specifically by the afamin gene. Because the genetic variants are assumed to act through afamin concentrations, the expected effects are probably small and not likely to be identified in genome-wide association studies. To evaluate possible causal relationships in humans, further (genetic) investigations such as Mendelian randomization studies will be necessary and are planned in our laboratory.

The observed slight but significant age and sex dependency of afamin plasma concentrations in our largest study groups may reflect correlations between afamin and other age- and sex-relevant parameters, such as lipids and blood pressure, and stands in contrast to findings of a recently published investigation of afamin in age-balanced healthy blood donors reporting no correlation between afamin and age or sex.[14] These differences are likely because of different population sizes and study design.

The significant association observed between afamin and high-sensitivity CRP plasma concentrations raises an interesting issue on a possible acute-phase property of afamin. Inverse associations between afamin and inflammatory biomarkers, including high-sensitivity CRP, observed in our previous study in a small group of patients with various, including inflammatory, diseases caused us to postulate a negative acute-phase function for afamin.[14] Additional larger studies are necessary to clarify this issue.

Strengths and Limitations

The study at hand has several strengths: first of all, initial data from transgenic animals overexpressing human afamin were in line with epidemiological studies in humans. One-year-old transgenic animals and their control littermates differed in key features of metabolic syndrome. Interestingly, adolescent animals did not yet show these differences, which points to an influential effect of afamin in the development of the metabolic features and which requires a certain amount of time. The association data with pronounced effects in humans

Table 3. Poisson and Logistic Regression Analyses of Afamin for Metabolic Syndrome and Its Components at Follow-up, Adjusted for Age, Sex, and Medication at Baseline (Where Appropriate) and Baseline Value of the Respective Outcome Variable

	Bruneck		SAPHIR		Meta-analysis*	
	IRR/OR (95% CI)†	*P* Value	IRR/OR (95% CI)†	*P* Value	IRR/OR (95% CI)†	*P* Value
Primary analyses						
Quasi-Poisson regression						
No. of metabolic syndrome components	1.08 (1.05–1.11)	1.12×10^{-7}	1.08 (1.05–1.11)	1.55×10^{-9}	1.08 (1.06–1.10)	8.87×10^{-16}
Logistic regression for metabolic syndrome						
Metabolic syndrome AHA/NHLBI (yes/no)	1.59 (1.36–1.86)	7.08×10^{-9}	1.61 (1.42–1.83)	1.63×10^{-13}	1.60 (1.45–1.77)	6.56×10^{-21}
Metabolic syndrome AHA/NHLBI (yes/no) (excluding those with metabolic syndrome at baseline)	1.77 (1.43–2.21)	2.33×10^{-7}	1.84 (1.57–2.16)	5.97×10^{-14}	1.82 (1.60–2.07)	7.99×10^{-20}
Metabolic syndrome AHA/NHLBI (yes/no) (excluding those with metabolic syndrome+diabetes mellitus at baseline plus diabetes mellitus at follow-up)	1.78 (1.42–2.24)	6.94×10^{-7}	1.83 (1.56–2.15)	2.24×10^{-13}	1.81 (1.59–2.07)	8.47×10^{-19}
Metabolic syndrome IDF (yes/no)	1.52 (1.26–1.84)	1.30×10^{-5}	1.59 (1.41–1.78)	5.39×10^{-15}	1.57 (1.42–1.73)	3.73×10^{-19}
Secondary analyses						
Logistic regression for components of the metabolic syndrome						
Hypertension (yes/no)	1.23 (1.07–1.42)	0.003	1.23 (1.11-0.1.37)	6.87×10^{-5}	1.23 (1.14–1.34)	7.10×10^{-7}
Elevated waist circumference (yes/no)	1.49 (1.25–1.79)	1.44×10^{-5}	1.44 (1.25–1.66)	3.74×10^{-7}	1.46 (1.31–1.63)	2.84×10^{-11}
Reduced HDL (yes/no)	1.39 (1.19–1.62)	2.02×10^{-5}	1.15 (0.97–1.36)	0.10	1.27 (1.05–1.53)	0.013
Elevated fasting triglycerides (yes/no)	1.28 (1.12–1.47)	3.88×10^{-4}	1.21 (1.09–1.35)	6.54×10^{-4}	1.24 (1.14–1.35)	1.05×10^{-6}
Elevated fasting glucose (yes/no)	1.34 (1.17–1.53)	2.10×10^{-5}	1.57 (1.41–1.76)	2.08×10^{-15}	1.46 (1.24–1.71)	5.13×10^{-06}

AHA indicates American Heart Association; CI, confidence interval; HDL, high-density lipoprotein; IDF, International Diabetes Foundation; IRR, incidence rate ratio; KORA, KOoperative Gesundheitsforschung in der Region Augsburg; NHLBI, National Heart, Lung, and Blood Institute; OR, odds ratio; and SAPHIR, Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk.

*Meta-analysis results based on a random-effects model.

†Afamin increment is 10 mg/L.

were in perfect agreement with the animal data and were derived from 3 independent populations, each showing the same effects for each of the metabolic syndrome components. Finally, the data were not derived only from cross-sectional analysis. Afamin was even associated with changes in number of metabolic syndrome components during a 5-year prospective observational period and thus with the development of metabolic syndrome in those free of the syndrome at baseline.

This study has also some limitations: we have limited data from only a small group of transgenic animals; a comprehensive phenotype analysis is still lacking. Therefore, this part of the work can be considered a pilot study. Furthermore, in our large human populations, we investigated only whites and it remains to be shown whether these findings can be confirmed in other ethnicities.

Various metabolic syndrome definitions are in place using various cut points and components. Because associations were also found in the linear regression analyses using the continuous variables for the respective metabolic syndrome components, it can be assumed that alternative cut points (eg, for waist circumference or glucose concentrations) provide comparable results, as shown by comparing analyses according to American Heart Association and the National Heart, Lung, and Blood Institute and International Diabetes Foundation definitions of metabolic syndrome (Tables 2 and 3).

Conclusions

Afamin concentrations were found to be strongly and positively associated with metabolic syndrome and all its individual components. This association is strengthened by the agreement observed between experimental studies in afamin-transgenic animals and epidemiological studies in 3 independent populations. The association was observed in cross-sectional, as well as prospective analyses. Early detection of a developing metabolic syndrome by measuring afamin might represent an important step in both the prevention and the treatment of this pandemic disease.

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Disclosures

Dr Dieplinger is owner and shareholder of Vitateq Biotechnology GmbH, a spin-off company of Innsbruck Medical University, holding

several patents related to research described in this article. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

The metabolic syndrome is defined as the coexistence of several risk factors for both type 2 diabetes mellitus and cardiovascular disease. It is associated with as the risk of developing type 2 diabetes mellitus and cardiovascular diseases. The search for key factors causing metabolic syndrome is mandatory for the identification of patients who need aggressive lifestyle modification focusing on weight-loss and physical activity. Afamin is a vitamin E–binding glycoprotein from human plasma primarily expressed in the liver and secreted into the bloodstream. On the basis of results of a study in transgenic mice overexpressing the human plasma protein afamin and revealing a metabolic-syndrome-like phenotype, we investigated a possible association between circulating afamin levels and metabolic syndrome in 3 independent, human population-based cohorts comprising >5000 white participants of European ancestry. Plasma afamin concentrations were found to be consistently associated with both the prevalence and the incidence of metabolic syndrome. In particular, the association between afamin levels and metabolic syndrome was observed for all components of the syndrome. In summary, results from our mouse model and the prospectively designed human studies suggest a potentially causal association between circulating afamin levels and the metabolic syndrome.

SUPPLEMENTAL MATERIAL

Laboratory measurements

In the Bruneck Study lipid parameters (total cholesterol (TC) and HDL cholesterol (HDLC)) as well as triglycerides (TG) were determined enzymatically (CHOD-PAP and GPO-PAP method, Merck, Darmstadt, Germany). LDL cholesterol (LDLC) was calculated with the Friedewald formula. Plasma glucose was analyzed colorimetrically using hexokinase and glucose-6-phosphate dehydrogenase. For experiment details, see Willeit and Kiechl.[1] Concentrations of high-sensitivity CRP (hs-CRP) were determined with an immunoturbidimetric latex CRP assay (OLYMPUS OSR6199; Olympus Diagnostics, Melville, NY, USA) using an IFCC/BCR/CAP CRM 470 CRP standard.[2]

In the SAPHIR Study plasma fasting glucose and a complete lipid profile including fasting TC, TG, HDLC and LDLC were determined using routine laboratory procedures (Roche Diagnostics GmbH, Mannheim, Germany). Hs-CRP was measured with the Tina-quant Cardiac C-reactive Protein (Latex) High Sensitive kit from Roche Diagnostics, Mannheim, Germany, on a Hitachi 911Chemistry Analyzer.[3]

In the KORA F4 Study TC was determined with the cholesterol-esterase method (CHOL Flex, Dade-Behring, Germany), whereas TG and HDLC were determined using the TGL Flex and the AHDL Flex methods (Dade-Behring), respectively. LDLC was measured with a direct method (ALDL, Dade-Behring).[4] Concentrations of high-sensitivity CRP (hs-CRP) were determined with nephelometry on a BN II using reagents from Siemens (Eschborn, Germany).[5]

Supplementary Table 1. Distribution of demographic parameters, vascular risk factors, other laboratory variables and metabolic syndrome components according to tertiles of afamin in the Bruneck (A), SAPHIR (B) and KORA F4 (C) study groups

	(A) Tertile groups of afamin: Bruneck Study (n=826)			
	Low (≤60.0) n=377	**Medium (>60.0-72.6) n=257**	**High (>72.6) n=192**	**P**
Age, yrs	57.9±11.2	57.9±11.2	58.1±10.9	0.970
Sex (Men/Women), %	(50.1/49.9)	(45.1/54.9)	(56.8/43.2)	0.051
Smoking (Non-smoker/ Ex-smoker/ Smoker), %	[58.6/24.4/17.0]	[52.5/26.1/21.4]	[50.0/28.1/21.9]	0.286
Waist circumference, cm	86.1±10.1	91.2±10.5	96.9±10.0	<0.001
Body mass index, kg/m²	24.1±3.3	26.1±3.6	28.2±3.7	<0.001
Obesity (BMI ≥30), %	3.7	16.0	31.3	<0.001
Total cholesterol, mg/dL	219.5±38.0	238.2±44.0	237.9±43.8	<0.001
LDL cholesterol, mg/dL	137.5±33.8	152.6±40.4	151.1±38.2	<0.001
HDL cholesterol, mg/dL	62.1±17.3	57.8±15.0	52.3±13.3	<0.001
Triglycerides, mg/dL	98.9±44.9 [70/90/115.5]	144.8±90.1 [91/129/164.5]	181.3±93.3 [119/160.6/218.3]	<0.001
hs-CRP, mg/L	3.6±9.4 [0.7/1.5/2.9]	3.1±5.5 [0.9/1.8/3.3]	3.4±4.8 [1.0/1.9/4.0]	0.004
Fasting glucose, mg/dL	97.3±16.8 [89.5/96/102]	102.6±22.4 [91/98/108]	111.9±34.8 [95/104/118]	<0.001
Diabetes mellitus, %	4.8	10.9	23.4	<0.001
Systolic BP, mmHg	143.4±19.5	150.4±20.5	154.8±21.1	<0.001
Diastolic BP, mmHg	84.7±8.8	88.2±9.1	90.2±8.9	<0.001
Metabolic syndrome and individual components				
Metabolic syndrome, %	14.6	38.9	59.4	<0.001
Hypertension, %	79.3	86.0	94.8	<0.001
Elevated waist circ. %	16.4	33.9	47.9	<0.001
Reduced HDL chol., %	11.7	20.2	30.2	<0.001
Elevated triglycerides, %	11.7	34.6	56.3	<0.001
Elevated glucose, %	31.3	43.6	58.3	<0.001
Number of metabolic syndrome components at baseline				
Entire group	1.5 ± 1.0	2.2 ± 1.2	2.9 ± 1.2	<0.001
Those with met. syndrome	3.2 ± 0.4	3.4 ± 0.7	3.7 ± 0.7	<0.001
Those without met. syndrome	1.2 ± 0.7	1.4 ± 0.7	1.7 ± 0.5	<0.001

Data are presented as mean ± SD and [25th, 50th and 75th percentile] in case of non-normal distribution or (%) if not indicated otherwise. The one-way ANOVA or Kruskal-Wallis test was applied for continuous variables, where appropriate. Dichotomized variables were compared using Pearson's χ^2 test. Tertile groups of afamin were calculated based on pooled data from the Bruneck, SAPHIR and KORA studies.

Supplementary Table 1. Continued

	(B) Tertile groups of afamin: SAPHIR Study (n=1499)			
	Low (≤60.0) n=545	**Medium (>60.0-72.6) n=547**	**High (>72.6) n=547**	**P**
Age, yrs	50.1±6.1	51.3±6.0	51.7±5.9	<0.001
Sex (Men/Women), %	[67.5/32.3]	[66.5/33.5]	[68.3/31.7]	0.845
Smoking (Non-smoker/ Ex-smoker/ Smoker), %	[68.1/10.8/21.1]	[64.2/14.1/21.8]	[57.7/19.2/23.1]	0.003
Waist circumference, cm	88.7±10.3	95.3±11.0	102.0±11.9	<0.001
Body mass index, kg/m²	24.6±2.9	26.9±3.8	29.5±4.1	<0.001
Obesity (BMI ≥30), %	3.9	16.7	41.3	<0.001
Total cholesterol, mg/dL	217.3±37.5	232.2±39.2	231.2±37.5	<0.001
LDL cholesterol, mg/dL	137.5±35.1	149.9±35.5	148.3±34.6	<0.001
HDL cholesterol, mg/dL	62.8±16.2	59.2±14.9	54.1±13.9	<0.001
Triglycerides, mg/dL	94.1±60.6 [57/77/106]	132.4±99.6 [78/105/152]	160.1±92.6 [100/135/187]	<0.001
hs-CRP, mg/L	2.8±9.6 [0.6/1.2/2.2]	2.7±4.1 [0.8/1.4/3.0]	3.1±3.1 [1.2/2.1/3.7]	<0.001
Fasting glucose, mg/dL	88.6±9.4 [83/88/94]	93.0±17.4 [85/91/97]	100.5±24.4 [89/96/103]	<0.001
Diabetes mellitus, %	0.6	2.2	8.1	<0.001
Systolic BP, mmHg	133.5±16.1	139.7±17.6	143.6±17.7	<0.001
Diastolic BP, mmHg	82.9±11.7	87.5±11.6	89.8±11.2	<0.001
Metabolic syndrome and individual components				
Metabolic syndrome, %	8.3	22.9	47.6	<0.001
Hypertension, %	64.8	78.7	87.4	<0.001
Elevated waist circ., %	16.1	40.3	65.7	<0.001
Reduced HDL chol., %	8.3	8.0	16.8	0.006
Elevated triglycerides, %	11.0	26.0	43.3	<0.001
Elevated glucose, %	9.9	19.4	35.3	<0.001
Number of metabolic syndrome components at baseline				
Entire group	1.09±0.96	1.72±1.12	2.48±1.12	<0.001
Those with met. syndrome	3.30±0.60	3.31±0.57	3.46±0.64	0.030
Those without met. syndrome	0.89±0.70	1.24±0.73	1.58±0.60	<0.001

Data are presented as mean ± SD and [25th, 50th and 75th percentile] in the case of non-normal distribution or (%) if not indicated otherwise. The one-way ANOVA or Kruskal-Wallis test was applied for continuous variables, where appropriate. Dichotomized variables were compared using Pearson's χ^2 test. Tertile groups of afamin were calculated based on pooled data from the Bruneck, SAPHIR and KORA studies.

Supplementary Table 1. Continued

	(C) Tertile groups of afamin: KORA Study (n=3060)			
	Low (≤60.0) n=855	**Medium (>60.0-72.6) n=976**	**High (>72.6) n=1229**	**P**
Age, yrs	52.0±13.8	55.9±13.3	59.0±12.0	<0.001
Sex (Men/Women), %	[43.6/56.4]	[48.1/51.9]	[51.7/48.3]	0.001
Smoking (Non-smoker/ Ex-smoker/ Smoker), %	[46.7/34.1/19.2]	[42.2/38.2/19.7]	[44.0/40.5/15.6]	0.008
Waist circumference, cm	86.5±12.0	92.6±13.3	100.0±13.2	<0.001
Body mass index, kg/m²	25.2±3.9	27.2±4.4	29.7±4.8	<0.001
Obesity (BMI ≥30), %	10.2	21.5	42.1	<0.001
Total cholesterol, mg/dL	204.3±37.0	215.8±37.4	224.0±40.9	<0.001
LDL cholesterol, mg/dL	126.4±33.0	136.2±34.0	142.5±35.2	<0.001
HDL cholesterol, mg/dL	58.5±14.2	57.2±14.7	52.9±13.8	<0.001
Triglycerides, mg/dL	88.9±71.4 [56/75/105]	112.5±59.3 [71/101/138]	159.7±105.4 [97/135/189]	<0.001
hs-CRP, mg/L	2.6±8.3 [0.4/0.9/1.9]	2.3±3.7 [0.6/1.2/2.5]	2.6±3.3 [0.8/1.5/3.1]	<0.001
Fasting glucose, mg/dL	91.5±12.1 [84/90/96]	95.7±15.4 [87/93/100]	105.0±23.6 [92/99/110]	<0.001
Diabetes mellitus, (%)	3.4	4.9	11.2	<0.001
Systolic BP, mmHg	117.0±17.6	121.5±18.7	126.4±18.0	<0.001
Diastolic BP, mmHg	72.7±9.5	74.7±9.9	77.1±9.9	<0.001
Metabolic syndrome and individual components				
Metabolic syndrome, %	11.5	22.7	48.9	<0.001
Hypertension, %	35.6	46.8	64.5	<0.001
Elevated waist circ., %	22.2	39.5	64.3	<0.001
Reduced HDL chol., %	16.3	17.8	25.3	<0.001
Elevated triglycerides, %	8.9	20.3	41.0	<0.001
Elevated glucose, %	17.4	26.7	49.3	<0.001
Number of metabolic syndrome components at baseline				
Entire group	0.99±1.10	1.51±1.30	2.45±1.42	<0.001
Those with met. syndrome	3.27±0.52	3.43±0.64	3.67±0.75	<0.001
Those without met. syndrome	0.70±0.76	0.94±0.80	1.27±0.77	<0.001

Data are presented as mean ± SD and [25th, 50th and 75th percentile] in the case of non-normal distribution or (%) if not indicated otherwise. The one-way ANOVA or Kruskal-Wallis test was applied for continuous variables, where appropriate. Dichotomized variables were compared using Pearson's χ^2 test. Tertile groups of afamin were calculated based on pooled data from the Bruneck, SAPHIR and KORA studies.

Distribution of measured parameters in subjects with vs without available afamin values

In the Bruneck Study 826 subjects participated in the 1995 examinations providing the basis for the current analyses. For all of those subjects afamin data were available; therefore there were no missing afamin data.

In the SAPHIR Study we compared those participants with and without afamin data (Supplementary Table 2). Those without afamin had been more often been diagnosed with "elevated waist circumference". This seems to be only based on a marginal difference in waist circumference in men. That is moreover the reason why the overall difference in metabolic syndrome is significantly different between the two groups. However, if we had afamin data of these subjects as well, our association of afamin with the component "elevated waist circumference" would have been even stronger.

In KORA we had afamin values from almost all subjects (3060 of 3080). Since only SAPHIR had a 15% portion of the cohort with missing afamin levels, we do not think that this could have influenced our major findings.

Supplementary Table 2. Distribution of demographic parameters and metabolic syndrome in those with vs. those without available afamin concentration in the SAPHIR study group

	SAPHIR with afamin (n=1499)	**SAPHIR without afamin (n=271)**	**P**
Age, yrs	51.0±6.0	53.7±5.4	<0.001
Sex (men/women), %	(67/36)	(33/64)	<0.001
Smoking (non-smoker/ ex-smoker/ smoker), %	(64/14/22)	(68/16/17)	0.189
Body mass index, kg/m²	26.8±4.1	26.9±4.3	0.722
Systolic BP, mmHg	138±18	141±20	0.030
Diastolic BP, mmHg	86±12	87±11	0.628
Waist circumference, cm (male)	97.8±10.5	100.4±10.5	0.020
Waist circumference, cm (female)	88.5±13.2	89.8±12.2	0.263
HDL cholesterol, mg/dL (male)	55.3±13.6	52.7±11.2	0.067
HDL cholesterol, mg/dL (female)	67.1±16.2	68.0±16.6	0.495
Triglycerides, mg/dL	126.0±89.3	123.0±78.3	0.990
Fasting glucose, mg/dL	93.4±18.0	93.9±16.3	0.392
Diabetes mellitus, %	3.2	3.7	0.678
Fibrates, %	0.2	1.1	0.049
Diabetes medication, %	1.5	1.1	0.786
BP medication, %	14.2	20.4	0.009
Metabolic syndrome, %	24.3	30.9	0.023
Hypertension, %	76.0	78.5	0.369
Elevated waist circumference. %	38.6	51.3	<0.001
Reduced HDL cholesterol, %	10.5	14.1	0.081
Elevated triglycerides, %	25.3	24.2	0.696
Elevated glucose, %	20.3	24.9	0.086

Data are presented as mean ± SD or (%) if not indicated otherwise.

Supplementary Table 3. Correlations between afamin and clinical variables, age, sex and smoking status in the Bruneck, SAPHIR AND KORA F4 studies

	Correlation coefficient (*r*)		
	Bruneck	**SAPHIR**	**KORA F4**
Age	0.001	0.119 [c]	0.227 [c]
Sex (1=female, 2=male)	-0.024	-0.004	-0.081 [c]
Smoking (0=non-smoker, 1=ex-smoker, 2=smoker)	0.056 [a]	0.05 [a]	0.009
Waist circumference	0.403 [c]	0.443 [c]	0.443 [c]
HDL cholesterol	-0.223 [c]	-0.232 [c]	-0.199 [c]
Triglycerides	0.495 [c]	0.422 [c]	0.468 [c]
Glucose	0.286 [c]	0.338 [c]	0.394 [c]
Systolic blood pressure	0.244 [c]	0.250 [c]	0.253 [c]
Diastolic blood pressure	0.271 [c]	0.258 [c]	0.207 [c]
hs-CRP	0.108 [b]	0.222 [c]	0.213 [c]
Diabetes mellitus (1=yes, 0=no)	0.265 [c]	0.176 [c]	0.173 [c]

Correlations between afamin and clinical continuous variables including components of metabolic syndrome and age were assessed with Spearman's rank correlation coefficient, correlations between afamin and binary variables (sex and diabetes mellitus) with the point-biserial correlation coefficient and between afamin and smoking with Kendall's tau correlation coefficient. [a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$.

Supplementary Table 4A: Poisson and logistic regression analysis of afamin for metabolic syndrome and its components at baseline, adjusted for age, sex, smoking status and ln-hsCRP

	Bruneck		SAPHIR		KORA F4	
	IRR / OR (95% CI) [b]	p	IRR / OR (95% CI) [b]	p	IRR / OR (95% CI) [b]	p
Primary analyses						
Quasi-Poisson regression for						
Number metabolic syndrome components	1.18 (1.15-1.20)	$8.96x10^{-50}$	1.19 (1.16-1.21)	3.0610^{-62}	1.16 (1.15-1.18)	$2.42x10^{-119}$
Logistic regression for metabolic syndrome						
Metabolic syndrome AHA/NHLBI (yes/no)	1.95 (1.72-2.21)	$6.65\ x10^{-25}$	1.80 (1.63-1.99)	$4.41x10^{-31}$	1.65 (1.55-1.75)	$8.15x10^{-62}$
Secondary analyses						
Logistic regression for components of the metabolic syndrome						
Hypertension (yes/no)	1.46 (1.25-1.70)	$1.30x10^{-6}$	1.37 (1.24-1.52)	$2.85x10^{-09}$	1.23 (1.17-1.30)	$1.27x10^{-14}$
Elevated waist circumference (yes/no)	1.76 (1.55-2.00)	$2.45x10^{-18}$	2.02 (1.81-2.24)	$3.01x10^{-38}$	1.59 (1.50-1.68)	$6.40x10^{-57}$
Reduced HDL (yes/no)	1.34 (1.19-1.52)	$1.32x10^{-6}$	1.19 (1.06-1.33)	0.0030	1.18 (1.12-1.24)	$8.09x10^{-10}$
Elevated fasting triglycerides (yes/no)	1.90 (1.68-2.16)	$5.34x10^{-24}$	1.58 (1.44-1.73)	$1.60x10^{-22}$	1.58 (1.49-1.67)	$7.95x10^{-56}$
Elevated fasting glucose (yes/no)	1.40 (1.26-1.55)	$1.08x10^{-10}$	1.49 (1.36-1.63)	$5.34x10^{-17}$	1.43 (1.35-1.51)	$1.43x10^{-38}$

[b] Increment for afamin is 10 mg/L

Supplementary Table 4B: Poisson and logistic regression analyses of afamin for metabolic syndrome and its components at follow-up, adjusted for age, sex, smoking status, ln-hsCRP and baseline value of the respective outcome variable

	Bruneck		SAPHIR	
	IRR / OR (95% CI) [b]	**p**	**IRR / OR (95% CI) [b]**	**p**
Primary analyses				
Quasi-Poisson regression for				
Number of metabolic syndrome components	1.07 (1.05-1.10)	$2.56x10^{-08}$	1.08 (1.05-1.11)	$1.92x10^{-09}$
Logistic regression for metabolic syndrome				
Metabolic syndrome AHA/NHLBI (yes/no)	1.66 (1.41-1.96)	$1.04x10^{-9}$	1.58 (1.39-1.80)	$4.39x10^{-12}$
Metabolic syndrome AHA/NHLBI (yes/no) (excluding those with metabolic syndrome at baseline)	1.83 (1.46-2.30)	$1.51x10^{-7}$	1.78 (1.51-2.09)	$4.19x10^{-12}$
Secondary analyses				
Logistic regression for components of the metabolic syndrome				
Hypertension (yes/no)	1.25 (1.08-1.44)	0.002552	1.22 (1.10-1.36)	$2.44x10^{-04}$
Elevated waist circumference (yes/no)	1.53 (1.27-1.83)	$5.66x10^{-6}$	1.38 (1.20-1.59)	$8.65x10^{-06}$
Reduced HDL (yes/no)	1.41 (1.21-1.65)	$1.25x10^{-5}$	1.15 (0.97-1.36)	0.1081
Elevated fasting triglycerides (yes/no)	1.28 (1.11-1.47)	$4.45\ x10^{-04}$	1.19 (1.06-1.33)	0.0028
Elevated fasting glucose (yes/no)	1.32 (1.15-1.51)	$5.37x10^{-5}$	1.55 (1.38-1.74)	$5.48x10^{-14}$

[b] Increment for afamin is 10 mg/L

Supplementary Table 5. Linear regression analysis of afamin on the continuous levels of parameters included in the metabolic syndrome definition at baseline, adjusted for age, sex and medication at baseline (where appropriate)

		Bruneck		SAPHIR		KORA F4		Meta-analysis [a]	
		IRR / OR (95% CI) [b]	p	IRR / OR (95% CI) [b]	p	IRR / OR (95% CI) [b]	p	IRR / OR (95% CI) [b]	p
Secondary analyses: Linear Regression for		**β (SE) [b]**	**p**	**β (SE) [b]**	**p**	**β (SE) [b]**	**p**	**β (SE) [b]**	**p**
Waist circumference	in men only	2.820 (0.249)	4.21×10^{-26}	3.352 (0.196)	7.98×10^{-58}	2.693 (0.153)	6.21×10^{-63}	2.951 (0.215)	1.09×10^{-42}
	in women only	2.998 (0.402)	5.27×10^{-13}	3.974 (0.428)	5.50×10^{-19}	2.971 (0.191)	1.10×10^{-50}	3.238 (0.293)	2.09×10^{-28}
HDL C	in men only	-1.898 (0.458)	4.12×10^{-5}	-2.522 (0.282)	1.62×10^{-18}	-1.244 (0.176)	2.62×10^{-12}	-1.868 (0.456)	4.26×10^{-5}
	in women only	-2.230 (0.583)	1.53×10^{-4}	-1.875 (0.568)	0.001	-1.633 (0.230)	1.95×10^{-12}	-1.733 (0.200)	4.80×10^{-18}
Systolic blood pressure		3.010 (0.427)	3.77×10^{-12}	2.139 (0.298)	1.11×10^{-12}	1.528 (0.180)	3.30×10^{-17}	2.151 (0.402)	8.83×10^{-08}
Diastolic blood pressure		1.507 (0.199)	9.14×10^{-14}	1.555 (0.204)	4.55×10^{-14}	1.144 (0.104)	7.01×10^{-28}	1.359 (0.149)	5.75×10^{-20}
Ln(triglycerides)		0.156 (0.010)	8.91×10^{-48}	0.152 (0.009)	9.90×10^{-54}	0.134 (0.005)	1.63×10^{-132}	0.145(0.008)	1.91×10^{-80}
Ln(glucose)		0.032 (0.003)	1.55×10^{-18}	0.028 (0.002)	2.24×10^{-32}	0.022 (0.001)	6.18×10^{-59}	0.027 (0.003)	4.70×10^{-19}

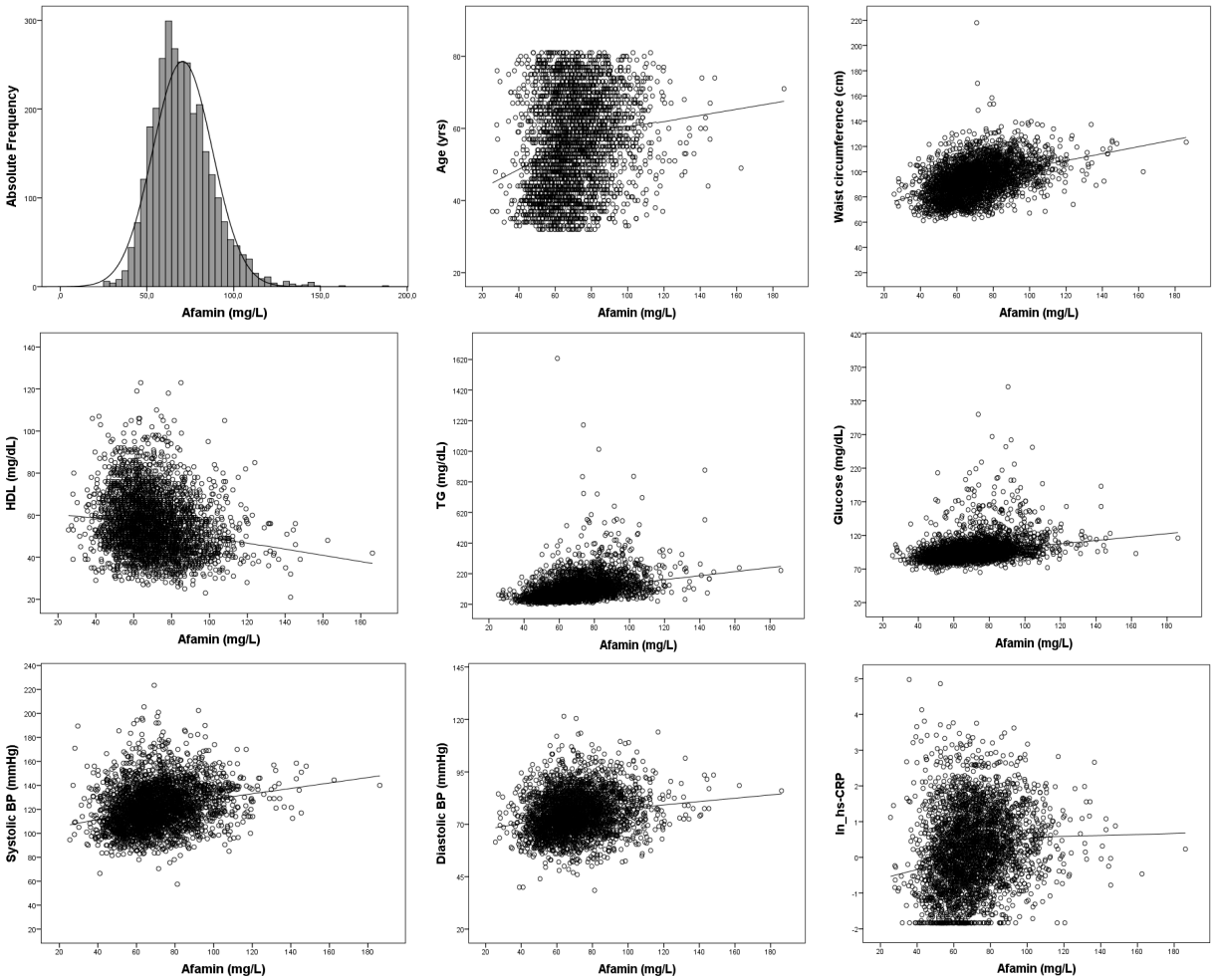
[a] Meta-analysis results based on a random-effects model. [b] Afamin's increment is 10 mg/L.

Supplementary Table 6. Linear regression analysis of afamin on the continuous levels of parameters included in the metabolic syndrome definition at follow-up, adjusted for age, sex and medication at baseline (where appropriate) and baseline value of the respective outcome variable

		Bruneck		SAPHIR		Meta-analysis [a]	
		IRR / OR (95% CI) [b]	**p**	**IRR / OR (95% CI) [b]**	**p**	**IRR / OR (95% CI) [b]**	**p**
Secondary analyses: Linear Regression for		**β (SE) [b]**	**p**	**β (SE) [b]**	**p**	**β (SE) [b]**	**p**
Waist circumference	in men only	-0.004 (0.195)	0.98	0.583 (0.140)	3.29×10^{-5}	0.305 (0.293)	0.2977
	in women only	0.529 (0.318)	0.10	0.855 (0.262)	0.001	0.723 (0.202)	0.0003
HDL C	in men only	-0.950 (0.313)	0.003	-0.750 (0.220)	0.001	-0.816 (0.180)	5.78×10^{-6}
	in women only	-1.010 (0.508)	0.05	-2.986 (0.431)	1.82×10^{-11}	-2.016 (0.988)	0.0412
Systolic blood pressure		0.219 (0.400)	0.58	0.221 (0.188)	0.24	0.221 (0.170)	0.19
Diastolic blood pressure		0.295 (0.195)	0.13	0.636 (0.302)	0.04	0.395 (0.164)	0.02
Ln(triglycerides)		0.030 (0.011)	0.006	0.016 (0.009)	0.08	0.022 (0.007)	0.002
Ln(glucose)		0.013 (0.004)	4.28×10^{-4}	0.014 (0.002)	6.37×10^{-9}	0.014 (0.002)	1.22×10^{-14}

For footnotes (a-b), see Table 2.

Supplementary Figure 1. Distribution of afamin and correlation plots for afamin with clinical continuous components of metabolic syndrome in the KORA F4 cohort



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