Plasma concentrations of afamin are associated with prevalent and incident type 2 diabetes: a pooled analysis in more than 20,000 individuals

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Abstract

Objective: The human vitamin E-binding glycoprotein afamin is primarily expressed in liver and has been associated with prevalent and incident metabolic syndrome. These data were in line with observations in transgenic mice. We thus investigated whether afamin concentrations are associated with prediabetes, type 2 diabetes, and insulin resistance.

Research Design and Methods: Individual-level baseline (n=20,136) and follow-up data (n=14,017) of 8 prospective cohort studies were investigated. Study-level data were combined using random-effects meta-analyses. Main outcomes were prevalent and incident type 2 diabetes, prediabetes, and insulin resistance. Discrimination and reclassification of participants was analysed for incident type 2 diabetes.

Results: Mean afamin concentrations between studies ranged from 61-73 mg/L. The eight studies included 1,398 prevalent and 585 incident cases of type 2 diabetes. Each increase of afamin by 10 mg/L was associated with prevalent type 2 diabetes: OR=1.19 (95%CI 1.12-1.26), $p=5.96x10^{-8}$. Afamin was positively associated with insulin resistance assessed by HOMA-IR: ß=0.110 (95%CI 0.089-0.132), $p=1.37x10^{-23}$. Most importantly, afamin measured at baseline was an independent predictor for 585 incident type 2 diabetes cases: OR=1.30 (95%CI 1.23-1.38), $p=3.53x10^{-19}$ and showed a significant and valuable gain in risk classification accuracy when added to this extended adjustment model.

Conclusions: This pooled analysis in more than 20,000 individuals showed that afamin is strongly associated with insulin resistance, prevalence and incidence of type 2 diabetes independent of major metabolic risk factors or parameters. Afamin might be a promising novel marker for the identification of individuals at high risk for the development of type 2 diabetes.

The worldwide number of adults with type 2 diabetes has quadrupled during the last 35 years. In 2014, the age-standardized prevalence rate was 9.0% for men and 7.9% for women, and is predicted to increase to 12.8% and 10.8%, respectively, by 2025 (1). Most importantly, about a third to a half of individuals with diabetes mellitus remains undiagnosed (2,3). Besides the enormous annual costs of 825 billion dollars worldwide, metabolic syndrome and diabetes mellitus increase subsequent non-fatal and fatal outcomes (2,4,5). More than 2 million deaths every year can be attributed to diabetes mellitus and its macrovascular and microvascular complications (1). Thus, an in-depth understanding of the pathogenesis as well as the identification of early risk predictors is of major importance.

We recently demonstrated in a pooled analysis of three epidemiological studies including more than 5,000 study participants that plasma afamin concentrations are predictive not only for the prevalence but also for the incidence of metabolic syndrome (6). In patients with polycystic ovary syndrome afamin concentrations have been reported to be associated with insulin resistance (7), but data on the association between afamin and type 2 diabetes are still lacking.

Afamin was first described in 1994 as the fourth member of the human albumin gene family including albumin, α-fetoprotein and vitamin D-binding protein (8,9). The human plasma glycoprotein afamin has a molecular mass of 87 kD with 15% carbohydrate content (10) and 55% amino acid sequence similarity to albumin (8). It is primarily expressed in the liver (8) but also in tissues such as brain, testes, ovaries and kidney (www.proteinatlas.org). Knowledge about the (patho-)physiological functions of this protein is still limited (11,12). Transgenic mice overexpressing the human afamin gene developed increased body weight and increased blood concentrations of lipids and glucose (6). Based on these findings and the epidemiological data on afamin and metabolic syndrome in humans (6), we aimed to investigate, whether afamin is associated with the prevalence and incidence of type 2 diabetes in a pooled analysis in more than 20,000 individuals from mainly population-based cohorts. Furthermore, we evaluated whether afamin is also related to prediabetes and type 2 diabetes-related phenotypes such as insulin resistance.

Research Design and Methods

Study Populations and Study Design

This investigation is based on eight prospective cohort studies, six of them were per definition population-based (Bruneck, KORA F3, KORA F4, CoLaus, YFS, and the NHLBI Family Heart Study), one study included unrelated healthy middle-aged men from nine general practices (NPHS-II), and one study was based on a healthy working population (SAPHIR). The baseline examination included a total of 20,136 individuals and from 14,017 individuals a follow-up examination was available. The baseline examination finally included a total of 20,094 individuals for prevalent and the follow-up examination 13,347 individuals for incident type 2 diabetes, respectively. Percentage of loss to follow-up varied between 3% (NPHS-II) and 36% (NHLBI Family Heart Study). This frequency could not be calculated for the CoLaus Study since follow-up collection of data on incident diabetes is still work in progress. The average follow-up time in the eight studies ranged from 4.5 to 12.5 years (Supplementary Table 1). All studies were approved by the respective local ethics committees. Clinical investigations described were carried out according to the Declaration of Helsinki. All participants provided written informed consent. For more details on study design, recruitment, clinical assessment of laboratory parameters and definition of outcomes see Supplementary Material.

Definition of outcomes

Type 2 diabetes was defined either as self-reported, and/or as fasting glucose ≥126 mg/dL, (≥7 mmol/L) according to the 1997 American Diabetes Association (ADA) criteria (13) and/or receiving anti-diabetic medication. Participants with diagnosis of type 1 diabetes were excluded. More details on the specific definitions in each study can be found in the Supplementary Material.

Measures of insulin resistance such as homeostasis model assessment-estimated insulin resistance (HOMA-IR) and whole-body insulin sensitivity index (ISI(composite)) were calculated as described in the Supplementary Material.

Prediabetes was specified according to the 1997 ADA definition (impaired fasting glucose defined as fasting glucose of ≥100-125 mg/dL (≥5.6-6.9 mmol/L) and impaired glucose tolerance as 2-h glucose value between ≥140-199 mg/dL (≥7.8-11.0 mmol/L)) (13).

Measurement of afamin plasma concentrations

Afamin was quantified with a custom-made double-antibody sandwich ELISA as previously described (6,10,14,15). Within-run and between-run coefficients of variation were 3.3% and 6.2%, respectively (15). Afamin concentrations were measured in all studies in the laboratory at the Medical University of Innsbruck. Extended information on the quality control of lab work is given in the Supplementary Material.

Statistical analyses in all cohorts

At baseline, the association between afamin and prevalent type 2 diabetes was explored by logistic regression analysis. At the follow-up investigation, logistic regression modelling of the relation of afamin values measured at baseline with incident type 2 diabetes was performed and participants with type 2 diabetes at baseline were excluded. Because exact dates of diagnosis of type 2 diabetes were not known in all studies, logistic instead of Cox proportional hazard regression was used for investigating incident type 2 diabetes. Both prevalent and incident type 2 diabetes were considered as primary outcomes. All further analysed outcomes (fasting insulin and glucose concentrations, glycated hemoglobin (HbA1c), HOMA-IR, whole-body ISI(composite) (in KORA F4 only)) were considered as secondary outcomes. For all analyses done, the first model was adjusted for age and sex and the second (referred to as extended adjustment model) additionally for other potential major metabolic risk factors or parameters (HDL cholesterol, triglycerides, BMI, hypertension and in 6 out of 8 studies glucose concentrations).

The linearity of afamin on all outcomes was tested by a penalized, age- and sex-adjusted regression spline approach in the large population-based in-house KORA F4 Study that served as a reference for all other studies included in the pooled analyses. In addition, results for afamin divided into quartiles are shown for primary outcomes.

Afamin concentrations are quite normally distributed (6). Whole-body ISI(composite), further continuous type 2 diabetes-related phenotypes (fasting insulin and glucose concentrations, HbA1c, HOMA-IR) and triglycerides were log-transformed based on the natural logarithm (ln) due to their skewed distribution.

To test heterogeneity between study-specific beta estimates, I^2 index as well as chi-square based Q-statistic was calculated for each outcome according to the age- and sex-adjusted model (16). Since there was an indication for heterogeneity for

prevalent diabetes (one of the two main outcomes) (Supplementary Table 2), a pooled effect size for the respective studies was calculated using random effects meta-analysis according to (17).

Further specific statistical analyses in the KORA F4 Study

For the primary outcome incident diabetes, both a model additionally including glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) beside major metabolic risk factors or parameters and a model considering glucose concentrations ≥100 mg/dL and family history of diabetes was calculated. This cut-off of ≥100 mg/dL for glucose concentrations was defined according to the 1997 ADA definition for impaired fasting glucose (IFG) (13).

Family history of diabetes in KORA F4 included information about diabetes for all first grade relatives and took age of onset into account (18). Variable selection in both adjustment models was based on the Framingham Risk Score for type 2 diabetes (19). Furthermore, logistic regression analyses were performed on the association of afamin with prediabetes and linear regression analyses on the association with whole-body ISI(composite). These latter analyses on whole-body ISI(composite) as well as linear regression models on further continuous type 2 diabetes-related phenotypes described above (fasting insulin and glucose concentrations, glycated hemoglobin (HbA1c), HOMA-IR) were calculated excluding participants with prevalent type 2 diabetes at baseline. HOMA-IR and whole-body ISI(composite) were also analysed divided by a cut-off of 2.5.

We considered incident type 2 diabetes as outcome also taking an oral glucose tolerance test (OGTT) into account and performed a test of deviances on nested models to assess whether afamin significantly added to the extended adjustment model. Whether afamin concentrations contributed to a better classification of individuals into predefined categories of incident type 2 diabetes risk in addition to a model already including major metabolic risk factors or parameters (age, sex, HDL cholesterol, triglycerides, BMI, hypertension and 1) fasting glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) or 2) fasting glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) and family history of diabetes was also evaluated. The categorical net reclassification improvement (NRI) was calculated using the reclass function in R based on the

following risk categories (<5%, 5-24% and >=25%) for individuals who developed type 2 diabetes during a median follow-up of 6.4 years (n=132) and for those who did not receive a diagnosis of type 2 diabetes (n=1,718) as well as for the total group. Standard errors for categorical NRI were computed according to Pencina et al. (20). For comparison purposes the continuous NRI was also calculated (again for cases and controls as well as the total group) with the function improveProb in R. The continuous NRI has the advantage over the categorical NRI that it does not depend on the choice of specific risk categories, and any change in predicted risk in the correct direction is considered appropriate.

For all analyses performed, a two-sided test P-value <0.05 was considered statistically significant. Analyses were performed using SPSS for Windows, version 21.0 (IBM Corp., Armonk, New York, NY, USA) and R for Windows, version 3.1.3 (Vienna, Austria).

Results

Baseline characteristics

Baseline characteristics of all eight studies included in this pooled analysis are shown in Supplementary Table 1. Mean afamin concentrations were lowest in the Young Finns Study (61.4±15.4 mg/L), and highest in the CoLaus Study (73.1±16.6 mg/L). Based on nonlinear P-splines there was no evident deviation from linearity of afamin in the applied regression models neither at baseline nor at follow-up in KORA F4 (Supplementary Figures 1 to 6). There was no effect of sex on associations of afamin with main outcomes (data not shown).

Association between afamin concentrations and prevalent type 2 diabetes (primary outcome)

The age- and sex-adjusted logistic regression analysis revealed an increased probability for prevalent type 2 diabetes per 10 mg/L increase in afamin concentrations (OR=1.40, 95%CI 1.31-1.48, $p=2.54x10^{-27}$). The extended model was additionally adjusted for HDL cholesterol, triglycerides, BMI and hypertension and still showed an OR=1.19, 95%CI 1.12-1.26, $p=5.96x10^{-08}$ (Figure 1, panel A and Supplementary Table 3). When afamin was categorized in quartiles, the association reached statistical significance in the age- and sex-adjusted model when the third and the fourth quartile

were compared to the first quartile (OR=1.74, 95%CI 1.38-2.20, $p=3.47x10^{-6}$ and OR=3.91, 95%CI 2.97-5.14, $p=2.10x10^{-22}$, respectively). This association was still significant for the fourth quartile after extended adjustment (OR=1.72, 95%CI 1.27-2.33, $p=5.09x10^{-4}$) (Figure 1, panel B, and Supplementary Table 4). In a sensitivity analysis we excluded the studies KORA-F3 and NPHSII from the pooled analysis since their participants were not necessarily fasting. This reduced heterogeneity, but led basically to the same results with slightly increased effect estimates.

Association between afamin concentrations and incident type 2 diabetes (primary outcome)

Afamin concentrations measured at baseline were also a significant predictor for the development of type 2 diabetes during follow-up. Each increase in afamin concentrations by 10 mg/L was significantly associated with a 49% higher odds for incident type 2 diabetes (OR=1.49, 95%CI 1.42-1.56, $p=5.97x10^{-62}$) in the age- and sex-adjusted model and with a 30% higher odds in the extended adjustment model (OR=1.30, 95%CI 1.23-1.38, $p=3.53x10^{-19}$) (Figure 2 panel A and Supplementary Table 3). When afamin concentrations were stratified in quartiles the association was most pronounced for the fourth quartile with an OR of 5.28 (95%CI 3.83-7.27, $p=2.64x10^{-24}$) in the age- and sex-adjusted model and an OR of 2.33 (95%CI 1.61-3.36, $p=6.66x10^{-6}$) in the extended adjustment model. This association was already present but less pronounced in the third quartile (age- and sex-adjusted: OR=2.56, 95%CI 1.88-3.49, $p=2.25x10^{-9;}$ extended adjustment model: OR=1.47, 95%CI 1.04-2.08, p=0.03) (Figure 2 panel B and Supplementary Table 5). Again, excluding KORA-F3 and NPHSII revealed similar results with slightly increased effect estimates.

Association between afamin concentrations and continuous type 2 diabetes-related phenotypes (secondary outcomes)

Further analyses on continuous type 2 diabetes-related phenotypes such as HbA1c, insulin, glucose and HOMA-IR were performed excluding all participants who already had type 2 diabetes at baseline. Baseline afamin concentrations were positively associated with insulin concentrations and HOMA-IR in the age- and sex-adjusted as well as in the extended adjustment model (Table 1 and Supplementary Table 6). An example of a forest plot is provided for HOMA-IR in Supplementary Figure 7. These associations were less pronounced but still statistically significant in both

adjustment models for glucose and HbA1c as dependent variables (Table 1 and Supplementary Table 6).

Extended analyses in the KORA F4 Study

Association between afamin and prediabetes as well as insulin resistance

Each increase of age- and sex-adjusted plasma afamin concentrations by 10 mg/L increased the probability for prediabetes based on the 1997 ADA definition in 2,635 KORA F4 individuals without type 2 diabetes at baseline: OR=1.41, 95%CI (1.33-1.49), $p=1.66x10^{-29}$. The same was observed for the extended adjustment model: OR=1.21, 95%CI (1.14-1.30), $p=8.62x10^{-09}$.

Besides these findings afamin was inversely related to insulin resistance based on whole-body insulin sensitivity index (ISI(composite)) in both adjustment models in the KORA F4 Study (Table 1). When this insulin resistance measure was stratified by a cut-off of 2.5, each increase in afamin concentrations by 10 mg/L was associated with an increased probability for insulin resistance (OR=1.89, 95%CI 1.67-2.15, $p=3.92x10^{-23}$). This association remained highly significant in the extended-adjustment model (OR=1.77, 95%CI 1.54-2.03), $p=6.94x10^{-16}$). The same association was found for HOMA-IR stratified by 2.5: each increase in afamin concentrations by 10 mg/L was related to a higher probability for insulin resistance in the age- and sex-adjusted model (OR=1.70, 95%CI 1.58-1.82, $p=5.91x10^{-91}$) and extended adjustment model (OR=1.47, 95%CI 1.34-1.56, $p=1.45x10^{-20}$), respectively.

Association between afamin and incident type 2 diabetes based on variable selection according to the Framingham Risk Score for type 2 diabetes

Further adjustment models on the development of type 2 diabetes were done. When fasting glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) were additionally included in the extended adjustment model, afamin concentrations measured at baseline were still a significant predictor for the development of type 2 diabetes (OR=1.35, 95%CI 1.17-1.57, $p=6.19x10^{-5}$). When all cohorts were taken into account where fasting plasma glucose concentrations were available, pooled effect estimates for afamin in these 6 studies did only marginally differ when compared to the single analysis in KORA F4 (with glucose concentrations as categorical variable (100-125 mg/dL vs. <100 mg/dL=reference) (OR=1.27, 95%CI

1.18-1.36, p=5.09*10^{-10}). Furthermore, when glucose concentrations were included in the model on a continuous scale, the effect estimate was almost unchanged (OR=1.21, 95%CI 1.11-1.30, p=2.87x10^{-6}) (for more details see Supplementary Table 7).

Even when besides glucose concentrations ≥100 mg/dL family history of diabetes was taken into account, each increase in afamin concentrations by 10 mg/L still showed a significantly higher probability for incident type 2 diabetes (OR=1.33, 95%CI 1.13-1.56, p=0.001).

Various further adjustment models for primary and secondary outcomes were done. No matter if we added either smoking, alcohol intake, physical activity, waist circumference (instead of BMI), family history of diabetes, fasting glucose concentrations, fasting insulin concentrations, or HOMA-IR (where appropriate) to the extended adjustment model, effect estimates of afamin remained highly significant (range of OR 1.20 to 1.43, all p values ≤0.001). Similar results were found for type 2 diabetes-related phenotypes which did not show major changes in the beta estimates for all outcomes (data not shown).

Afamin and type 2 diabetes risk discrimination and reclassification analysis

To assess whether afamin contributes to a better discrimination between individuals who developed type 2 diabetes and those who remained free of type 2 diabetes during the prospective follow-up in the KORA F4 Study, two statistical concepts were applied: 1) deviances and 2) categorical as well as continuous net reclassification index (NRI). For these analyses we applied a more accurate definition for incident type 2 diabetes available in KORA F4 further using an oral glucose tolerance test (OGTT) (according to the 1997 ADA criteria) (13). The effect estimate of afamin did not change compared to the diabetes definition without OGTT as used in the pooled analysis according to the extended adjustment model (OR=1.48, 95%CI 1.32-1.66, p= 5.96*10^{-11} vs. OR=1.40, 95%CI 1.23-1.60, p=5.49*10^{-7}). The model including afamin (deviance= 694.69) showed a significantly improved model fit compared to the extended risk model including glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) (deviance= 726.90) (difference in deviance -32.21, p<0.0001). When besides glucose concentrations ≥100 mg/dL family history of diabetes was additionally included in the extended adjustment model (deviance= 602.71), the model also containing afamin (deviance= 577.07) still indicated a significantly improved model fit (difference in deviance -25.64, p<0.0001).

Furthermore, the categorical NRI was applied to test whether inclusion of afamin into a model containing known metabolic risk factors or parameters significantly adds to type 2 diabetes risk reclassification. Based on predefined risk categories (<5%, 5-24%, ≥25%), as shown in Table 2, NRI for cases was 0.114 (95%CI 0.031-0.221), p=0.002 and for controls 0.021 (95%CI 0.006-0.036), p=0.008. Overall NRI for the total group was 0.135 (95%CI 0.048-0.221, p=0.002). Of the 132 individuals who developed type 2 diabetes, 24 (18.2%) were correctly reclassified and thus moved to a higher risk category. Of those who remained free of type 2 diabetes (n=1,718), 110 (6.4%) moved to a lower risk category and can be considered as correctly reclassified based on adding afamin to the risk model. In subjects at intermediate risk (5% to <24%), the addition of afamin to the risk model resulted in a correct reclassification of 17 cases (24.3%) and 84 controls (19.9%), respectively (Table 2 and Supplementary Figure 8). Even when additionally adding family history of diabetes to the risk model, afamin still contributed to an improved type 2 diabetes risk reclassification (see Supplementary Table 8 and Supplementary Figure 9). Results based on continuous NRI showed a significant gain in classification accuracy when afamin was added to the risk model: NRI for cases 0.197 (95%CI: 0.030-0.364) p=0.02, and for controls 0.354 (95%CI 0.310-0.398) p<0.0001. Overall continuous NRI for the total group was 0.551 (95%CI: 0.378-0.724), p<0.0001. This means that in about three of five subjects the assignment to the case or control status has been enforced by adding afamin to the risk model. The same conclusion holds true when also family history of diabetes was included in the risk classification calculations because absolute NRI values did not change for NRI for the total group 0.491 (95%CI: 0.298-0.685), p<0.0001 and NRI for controls 0.351 (95%CI: 0.305-0.398), p<0.0001, and were only slightly attenuated for NRI for cases 0.140 (95%CI: 0.047-0.328), p=0.14).

Conclusions

This is the first analysis in more than 20,000 individuals from mainly population-based studies that describes novel associations of afamin with prevalent and incident type 2 diabetes and type 2 diabetes-related phenotypes. The main findings were: 1) increased afamin concentrations were significantly associated with prediabetes and type 2 diabetes at baseline and type 2 diabetes-related phenotypes such as insulin

resistance defined by HOMA-IR and whole-body ISI(composite). 2) Afamin concentrations at baseline significantly predicted the development of type 2 diabetes during follow-up. All these associations were independent from major metabolic risk factors or parameters. 3) Afamin showed a significant improved model fit and gain in classification accuracy for incident type 2 diabetes when added to an extended adjustment model including major metabolic risk factors or parameters.

Previously, we showed that afamin concentrations measured at baseline were significantly related to all components of the metabolic syndrome, with one of the strongest associations found with elevated waist circumference at both the baseline and follow-up investigation (6). Elevated waist circumference and BMI are measures of increased body fat and well-established risk factors for the metabolic syndrome and type 2 diabetes (21-23). Furthermore, this increase in body fat elevates not only the risk for type 2 diabetes but also for insulin resistance. Most importantly, in our large analysis afamin was associated with prediabetes, measures of insulin resistance as well as the prevalence and incidence of type 2 diabetes independently of major metabolic factors or parameters. Taken together, the findings on incident type 2 diabetes and prediabetes strongly suggest that afamin might be a valid marker to predict a high risk for developing type 2 diabetes. Novel mechanisms and pathways besides those related to metabolic syndrome might be involved.

Adipose tissue can affect the development of insulin resistance in other tissues such as liver by producing free fatty acids and several other pro- and anti-inflammatory factors (24). Insulin resistance causes hyperinsulinemia and leads to steatosis via various mechanisms such as increased hepatic *de novo* lipogenesis (24), inflammation, and lipotoxicity (25). There is evidence that non-alcoholic fatty liver disease might also be a risk factor for future type 2 diabetes and not only *vice versa* (26). As afamin is primarily expressed in liver, the liver might indeed play an important role in contributing to elevated afamin concentrations and thus development of type 2 diabetes.

In general, afamin seems to have heterogeneous effects depending on the site of action. It has been shown that afamin might have binding properties for two of the major forms of anti-oxidative vitamin E, α-tocopherol and γ-tocopherol (14). The anti-oxidative function of vitamin E remains controversial (27). Our previous work has demonstrated that plasma afamin concentrations are not associated with those of

vitamin E, indicating that afamin does not play a major role in binding and transporting vitamin E in plasma (in fact, vitamin E is mostly carried by the lipoprotein system) (10). Thus, the proposed vitamin E binding role of afamin might be of functional relevance for diseases such as type 2 diabetes and metabolic syndrome only in extravascular fluids or tissues. Possible mechanisms for such a scenario remain unknown.

The causality of afamin's association with type 2 diabetes as well as possible underlying mechanisms remains to be elucidated. The preliminary findings of a hyperglycemic phenotype in mice transgenic for the human afamin gene are supportive for a causal role of afamin for the development of type 2 diabetes (6). A direct role of afamin in glucose metabolism was very recently shown by Shen et al. in a thyroid carcinoma cell line transfected with human afamin (28). Afamin was found to upregulate several key enzymes and metabolites of glucose metabolism revealing new possible insights into the molecular functions of afamin. Since the transgenic animals as well as the transfected cell line model are of only limited relevance for the pathogenesis of type 2 diabetes in humans, both models have to be considered with caution as valid models for a functional and causal role of afamin in type 2 diabetes.

Our results are in accordance with a recently reported study demonstrating a strong association between concentrations of microRNA-122 (miRNA-122), and the incidence for metabolic syndrome and type 2 diabetes in the Bruneck Study (29). MiRNA-122 was also highly significantly associated with afamin analysed by proteomics approach. MiRNAs play a key role in the epigenetic regulation of gene expression. MiRNA-122 is the predominant miRNA in liver and regulates a number of genes involved in cholesterol and fatty acid metabolism (for review, see (30). Willeit et al. therefore investigated in a mouse model the expressed hepatic proteome after antisense targeting of miRNA-122. Afamin was not differentially expressed when comparing untreated mice with mice lacking miRNA-122 suggesting no gene regulatory function of miRNA-122 for afamin at least in mice (29).

Finally, the question remains whether afamin adds information to well-known risk predictors for incident type 2 diabetes. All measures of discrimination and reclassification, i.e. deviance, continuous and categorical NRI, suggested a significant and valuable gain in model fit and classification accuracy in the population-based KORA F4 Study when afamin measured at baseline was added to a risk model including age, sex, metabolic risk factors or parameters, glucose concentrations ≥100

mg/dL and a positive family history of diabetes. This is even more impressive as most of these metabolic risk factors or parameters are major components of the metabolic syndrome.

A main strength of the study is that data were generated from eight independent populations, the great majority of them being population-based. In addition, we had follow-up data on incident type 2 diabetes available in all of these studies. It might be considered as a limitation that we performed the extended analyses and adjusted for potential confounders or risk factors such as smoking, alcohol intake, physical activity, waist circumference or fasting glucose concentrations and family history of diabetes mainly in the large population-based in-house KORA F4 Study that had all this variables available and included only fasting participants. However, a further analysis was added adjusting for fasting glucose concentrations in 6 of the 8 cohorts that had fasting glucose concentrations available, and results remained highly consistent. Data on family history of diabetes besides the power issue might be moreover susceptible to inaccuracies. However, doing so, showed very similar results as in the presented main pooled analyses.

Statistical concepts for risk reclassification such as categorical NRI have known limitations such as the arbitrary choice of risk categories if no recommended risk thresholds exist. Therefore, we also applied the continuous NRI that does not rely on predefined risk categories. Moreover, the result of the test on deviances was in line with the results of both NRI analyses. Thus, the model performance of afamin was consistent over all applied statistical concepts of risk prediction and discrimination. Marginal differences in NRI analyses when family history of diabetes was further added to the risk model were most probably caused by limited statistical power; however, the main conclusion drawn that afamin improved type 2 diabetes risk reclassification did not change. Moreover, as in most epidemiological studies, we cannot exclude that results are to some extent biased by residual and unmeasured confounding as well as loss-to-follow-up. Finally, the analyses were performed only in Caucasians and thus it has to be elucidated whether these findings can be replicated in other ethnicities.

In summary, this large analysis of mainly population-based studies demonstrated that afamin is highly significantly associated with prediabetes, insulin resistance, prevalence of type 2 diabetes as well as the development of type 2 diabetes independent of major metabolic risk factors or parameters. Increased plasma afamin

concentrations may therefore indicate the development of type 2 diabetes already at a very early stage. As the number of individuals diagnosed with diabetes is steadily increasing since decades and according to the WHO global diabetes prevalence has doubled since 1980, finding crucial markers contributing to the development of type 2 diabetes is indispensable for an adequate and rapid identification of affected patients or patients at high risk as well as for the elucidation of the pathogenesis of this disease.

Contributors

FK is the guarantor of this work. BK, CL, HD and FK designed the study. BK, CL, PMV, SK, IS, JC, SCH and FK did the analyses, interpreted the findings, and wrote and revised the report. BK, FK, CH, SK, IS, SCH, CM, CH, LK, JW, BT, DD, DS, KW, MR, WRa, BP, AP, MK, TL, OTR, SHE and PV were involved in the design, recruitment, phenotyping, data collection, data preparation, and data management of the singular cohorts. All authors contributed to critical reading and revision of the draft report.

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Declaration of interests

HD is owner and shareholder of Vitateq Biotechnology GmbH, a spin-off company of Medical University of Innsbruck, holding several patents related to research described in this article. All other authors disclose no conflicts of interest.

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Figures legends

Figure 1: Forest plot illustrating the association of afamin with prevalent type 2 diabetes (extended adjustment model), based on a random effects (RE) model for all 8 studies as well as excluding KORA F3 and NPHSII since most participants in these studies were non-fasting. Panel A provides data for an afamin increment of 10 mg/L and panel B provides data for afamin divided into quartiles. Odds Ratios and 95% confidence intervals are shown for each study and the pooled analyses. Numbers for prevalent type 2 diabetes (yes / no) refer to the age- and sex-adjusted model.

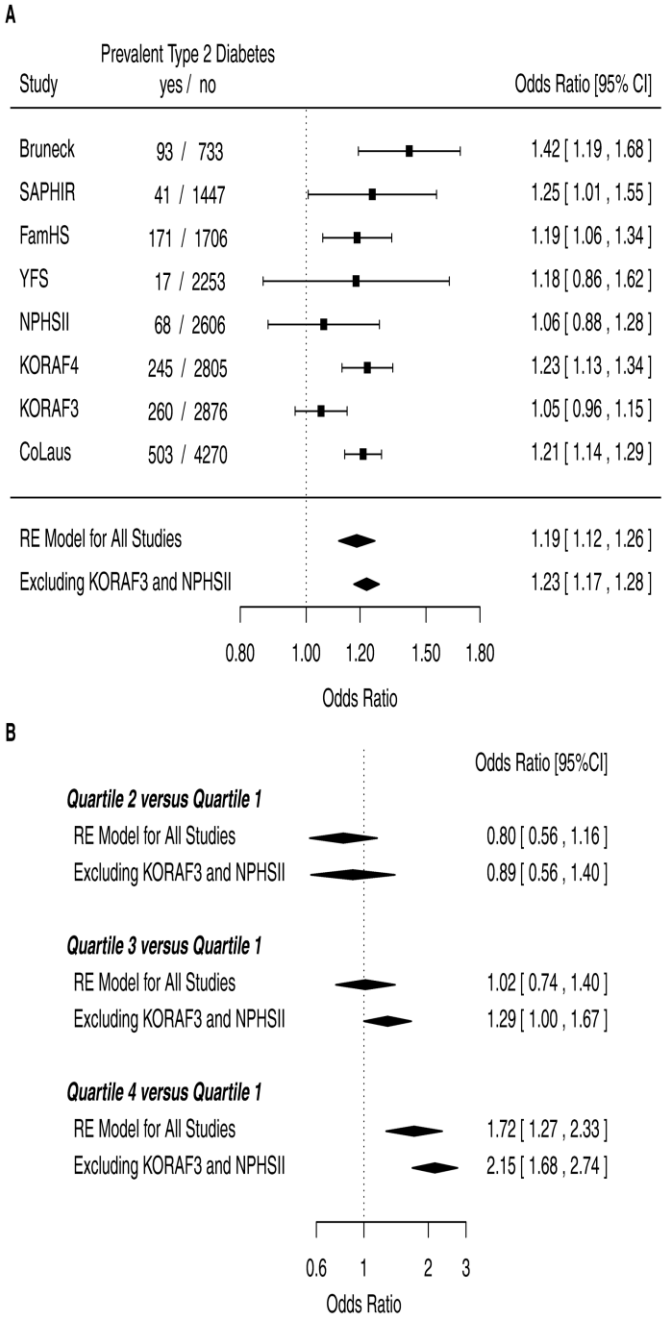

Figure 2: Forest plot illustrating the association of afamin (increment 10 mg/L) with incident type 2 diabetes (extended adjustment model), based on a random effects (RE) model for all 8 studies as well as excluding KORA F3 and NPHSII. Panel A provides data for an afamin increment of 10 mg/L and panel B for afamin divided into quartiles. Odds Ratios and 95% confidence intervals are shown for each study and the pooled analyses. Numbers for incident type 2 diabetes (yes / no) refer to the age- and sex-adjusted model.

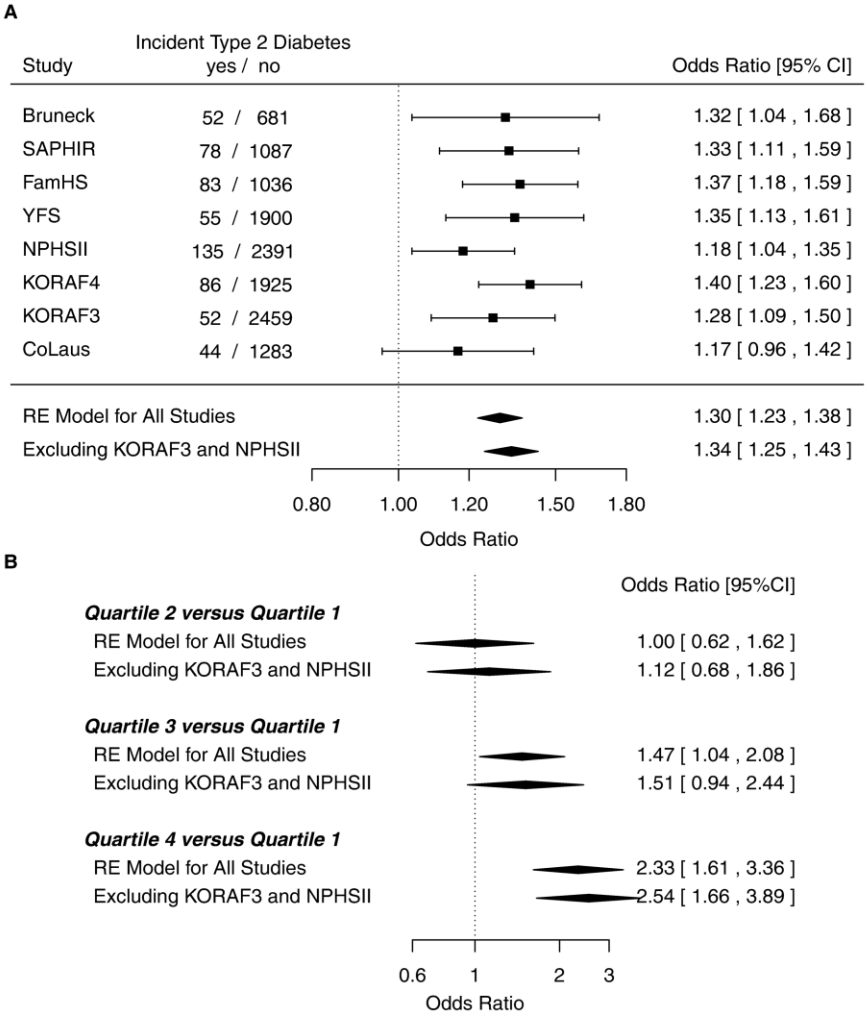

Table 1: Pooled results from study-specific linear regression analyses of afamin (increment 10 mg/L) on type 2 diabetes-related phenotypes at the baseline investigation excluding those with type 2 diabetes at baseline.

	Adjustment for age and sex		Extended adjustment	
Parameters / (n individuals)	**ß (95% CI)** [*, ‡]	**P**	**ß (95% CI)** [†, ‡]	**P**
Ln-HbA1c (**%**) (n=7,828) [§]	0.006 (0.004-0.008)	4.41×10^{-10}	0.003 (0.002-0.005)	3.09×10^{-4}
Ln-Insulin (µlU/ml) (n=13,156) [‖]	0.172 (0.146-0.198)	3.32×10^{-39}	0.101 (0.083-0.120)	1.51×10^{-26}
Ln-Glucose (mg/dL) (n=13,183) [‖]	0.015 (0.010-0.020)	4.68×10^{-10}	0.009 (0.006-0.013)	7.48×10^{-7}
Ln-HOMA-IR (n=13,153) [‖]	0.187 (0.158-0.216)	3.00×10^{-36}	0.110 (0.089-0.132)	1.37×10^{-23}
Ln-ISI(composite) (n=926) [¶]	-0.246 (-0.278- -0.214)	2.18×10^{-50}	-0.171 (-0.204- -0.137)	4.53×10^{-24}

N refer to the age- and sex-adjusted model; Ln refers to log-transformation based on the natural logarithm (ln).

[*] Adjusted for age and sex;

[†] Adjusted for age, sex, HDL cholesterol, triglycerides, BMI and hypertension

[‡] Meta-analysis beta estimate, 95% CI and P-values derived from a random effects model

[§] Studies included: Bruneck, SAPHIR, KORA F3, and KORA F4

[‖] Studies included: Bruneck, SAPHIR, KORA F4, CoLaus, YFS, and FamHS

[¶] Study included: KORA F4

Table 2: Reclassification of individuals into low, medium and high risk categories for development of type 2 diabetes within the study period in the KORA F4 Study (median follow-up 6.4 years) when additionally considering afamin in the risk model. The baseline model includes the risk factors or parameters age, sex, HDL cholesterol, triglycerides, BMI, hypertension and glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL= reference).

Individuals with incident type 2 diabetes (n=132)				
		Baseline model plus afamin		
Baseline model	Total	<5% risk	5-24% risk	>=25% risk
<5% risk	17	10 (58.8)	7 (41.2) *	0 (0.0) *
5-24% risk	70	4 (5.7) †	49 (70.0)	17 (24.3) *
>=25% risk	45	0 (0.0) †	5 (11.1) †	40 (88.9)
Total	132	14	61	57

* Moved to higher risk category which is correctly reclassified (light gray), n = 24; † Moved to lower risk category which is wrongly reclassified (dark gray), n = 9; stayed in the same risk category (medium grey), n=99; **NRI 0.114 (95%CI 0.031-0.221), p=0.002.**

Individuals without incident type 2 diabetes (n=1,718)				
		Baseline model plus afamin		
Baseline model	Total	<5% risk	5-24% risk	>=25% risk
<5% risk	1,202	1,156 (96.2)	45 (3.7) †	1 (0.08) †
5-24% risk	422	84 (19.9) *	310 (73.5)	28 (6.6) †
>=25% risk	94	0 (0.0) *	26 (27.7) *	68 (72.3)
Total	1,718	1,240	381	97

* Moved to lower risk category which is correctly reclassified (light gray), n =110; † Moved to higher risk category which is wrongly reclassified (dark gray), n=74; stayed in the same risk category (medium grey); n =1534; **NRI 0.021 (95%CI 0.006-0.036), p=0.008.**

Values are presented as n (row percent).

Categorical net reclassification improvement (NRI) in this table is calculated for 132 individuals with and for 1,718 individuals without type 2 diabetes. **Overall NRI for the total group: 0.135 (95%CI 0.048-0.221), p=0.002.**