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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.

Acknowledgments

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

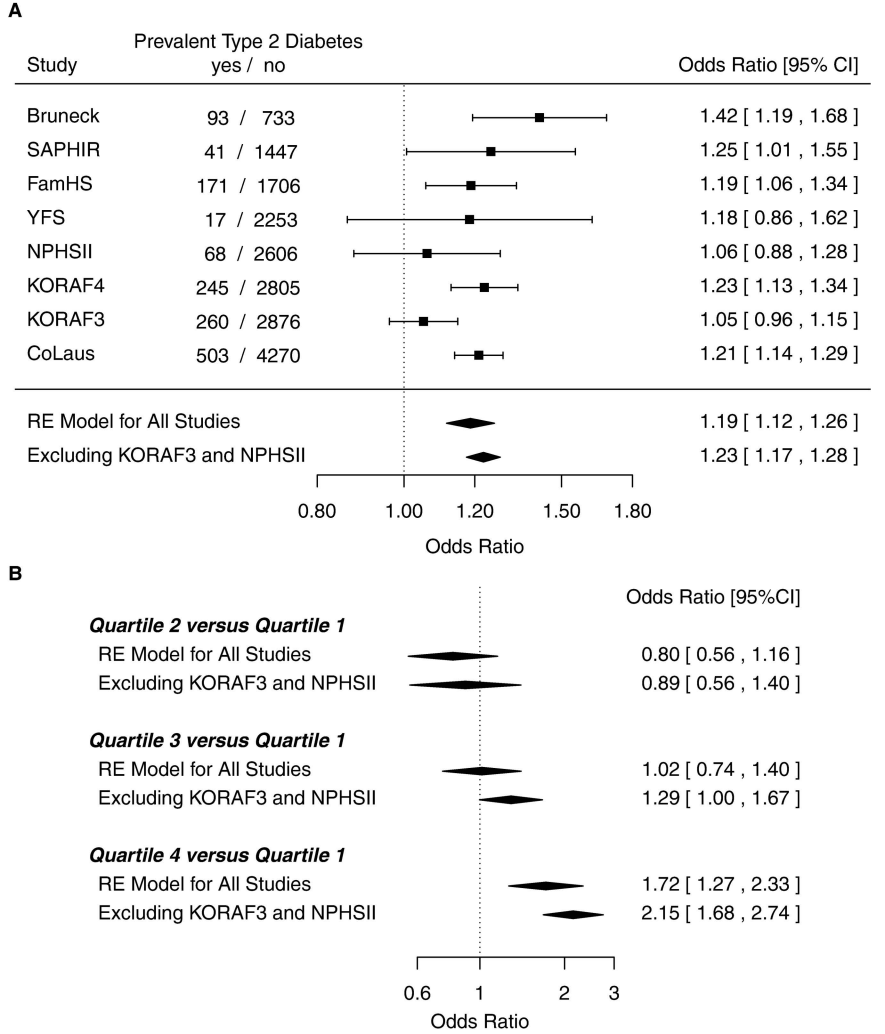
* Moved to higher risk category which is correctly reclassified (light gray), n = 24; † Moved to lower risk category which is wrongly reclassified (dark gray), n = 9; stayed in the same risk category (medium grey), n=99; **NRI 0.114 (95%CI 0.031-0.221), p=0.002.**

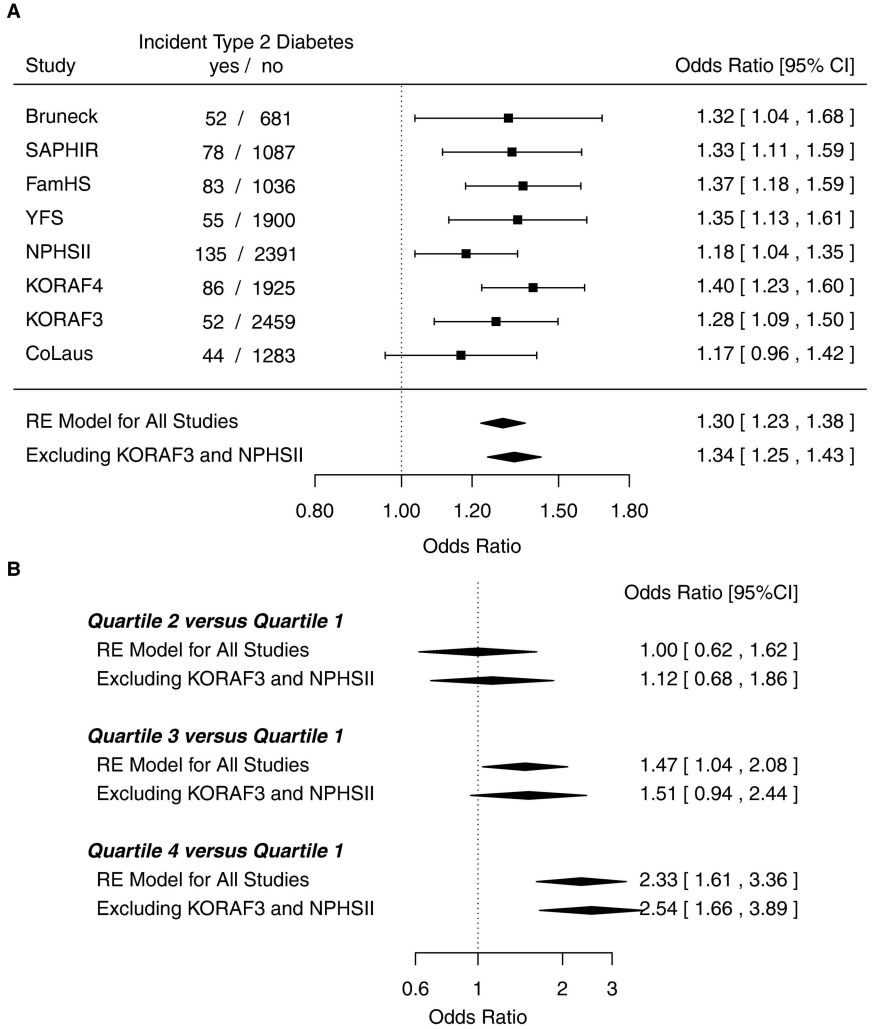
Individuals without incident type 2 diabetes (n=1,718)				
		Baseline model plus afamin		
Baseline model	Total	<5% risk	5-24% risk	>=25% risk
<5% risk	1,202	1,156 (96.2)	45 (3.7) †	1 (0.08) †
5-24% risk	422	84 (19.9) *	310 (73.5)	28 (6.6) †
>=25% risk	94	0 (0.0) *	26 (27.7) *	68 (72.3)
Total	1,718	1,240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


A
Study
Prevalent Type 2 Diabetes
yes / no
Odds Ratio [95% CI]
Bruneck
93 / 733
1.42 [1.19 , 1.68]
SAPHIR
41 / 1447
1.25 [1.01 , 1.55]
FamHS
171 / 1706
1.19 [1.06 , 1.34]
YFS
17 / 2253
1.18 [0.86 , 1.62]
NPHSII
68 / 2606
1.06 [0.88 , 1.28]
KORAF4
245 / 2805
1.23 [1.13 , 1.34]
KORAF3
260 / 2876
1.05 [0.96 , 1.15]
CoLaus
503 / 4270
1.21 [1.14 , 1.29]
RE Model for All Studies
1.19 [1.12 , 1.26]
Excluding KORAF3 and NPHSII
1.23 [1.17 , 1.28]
0.80
1.00
1.20
1.50
1.80
Odds Ratio
B
Odds Ratio [95%CI]
Quartile 2 versus Quartile 1
RE Model for All Studies
0.80 [0.56 , 1.16]
Excluding KORAF3 and NPHSII
0.89 [0.56 , 1.40]
Quartile 3 versus Quartile 1
RE Model for All Studies
1.02 [0.74 , 1.40]
Excluding KORAF3 and NPHSII
1.29 [1.00 , 1.67]
Quartile 4 versus Quartile 1
RE Model for All Studies
1.72 [1.27 , 2.33]
Excluding KORAF3 and NPHSII
2.15 [1.68 , 2.74]
0.6
1
2
3
Odds Ratio


A
Incident Type 2 Diabetes
Study
yes / no
Odds Ratio [95% CI]
Bruneck 52 / 681 1.32 [1.04 , 1.68]
SAPHIR 78 / 1087 1.33 [1.11 , 1.59]
FamHS 83 / 1036 1.37 [1.18 , 1.59]
YFS 55 / 1900 1.35 [1.13 , 1.61]
NPHSII 135 / 2391 1.18 [1.04 , 1.35]
KORAF4 86 / 1925 1.40 [1.23 , 1.60]
KORAF3 52 / 2459 1.28 [1.09 , 1.50]
CoLaus 44 / 1283 1.17 [0.96 , 1.42]
RE Model for All Studies 1.30 [1.23 , 1.38]
Excluding KORAF3 and NPHSII 1.34 [1.25 , 1.43]
0.80 1.00 1.20 1.50 1.80
Odds Ratio
B
Odds Ratio [95%CI]
Quartile 2 versus Quartile 1
RE Model for All Studies 1.00 [0.62 , 1.62]
Excluding KORAF3 and NPHSII 1.12 [0.68 , 1.86]
Quartile 3 versus Quartile 1
RE Model for All Studies 1.47 [1.04 , 2.08]
Excluding KORAF3 and NPHSII 1.51 [0.94 , 2.44]
Quartile 4 versus Quartile 1
RE Model for All Studies 2.33 [1.61 , 3.36]
Excluding KORAF3 and NPHSII 2.54 [1.66 , 3.89]
0.6 1 2 3
Odds Ratio

Supplementary Material to:

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

Barbara Kollerits [1], Claudia Lamina [1], Cornelia Huth [2,3], Pedro Marques-Vidal [4], Stefan Kiechl [5], Ilkka Seppälä [6,7], Jackie Cooper [8], Steven C. Hunt [9,10], Christa Meisinger [2,3], Christian Herder [3,11], Ludmilla Kedenko [12], Johann Willeit [5], Barbara Thorand [2,3], Doreen Dähnhardt [1], Doris Stöckl [2,3], Karin Willeit [5], Michael Roden [3,11,13], Wolfgang Rathmann [3,14], Bernhard Paulweber [12], Annette Peters [2,3,15], Mika Kähönen [16,17], Terho Lehtimäki [6,7], Olli T Raitakari [18,19], Steve E. Humphries [8], Peter Vollenweider [4], Hans Dieplinger [1,20], Florian Kronenberg [1]

Study Populations and Study Design

KORA F3 and KORA F4

The Cooperative Health Research in the Region of Augsburg (KOoperative Gesundheitsforschung in der Region Augsburg, KORA) Study incorporates **population-based cohort studies** drawn from equally sized ten year age-sex-strata of the target population which consists of all 25 to 74 year old German residents of the city of Augsburg, Germany and two surrounding counties, and was initiated as part of the WHO MONICA Study. A detailed description of the sampling methods is given elsewhere [(1)]. A standardized face-to-face interview and medical examinations including blood draw as well as anthropometric measurements were done by certified medical staff in all study participants [(1)]. Moreover, participants were asked to bring all product packages of currently used medication to the study centre.

The KORA F3 study is a follow-up investigation of the KORA S3 study conducted in 1994/1995 with a response rate of 75%. Of all 4,856 KORA S3 participants, 3,184 also participated in 2004/2005 in KORA F3. About 92% of the KORA F3 participants were non-fasting. Afamin data were available in 3,158 KORA F3 participants. Prevalent type 2 diabetes at KORA F3 was defined as self-reported and validated by hospital records or by questioning the responsible physician, or as current use of antidiabetic medication. Additionally, a validation of the diabetes type was requested. If no type validation, but also no contradicting information was given, diabetic participants were assumed to have type 2 diabetes.

Incident cases of type 2 diabetes were mainly assessed using follow-up questionnaire data collected in 2008/2009. Self-reported type 2 diabetes and the date of diagnosis were validated by hospital records or by questioning the responsible physician. Furthermore, hospital records of those deceased during the follow-up period were examined. The records were searched for a history of type 2 diabetes and the date of diagnosis. If a physician-diagnosis of type 2 diabetes was known from other sources, e.g. from the records of the population-based MONICA/KORA registry of acute myocardial infarction, this information was also used. In general, incident cases of type 2 diabetes, which had been diagnosed up to December 31, 2009, were included. In total, 13% of participants were lost to follow-up.

The KORA F4 study is a follow-up of the independent KORA S4 survey, conducted between 1999 and 2001 in the same geographical region as KORA S3, with a response rate of 67%. Of all 4,261 KORA S4 participants, 3,080 also participated between 2006 and 2008

in the follow-up study KORA F4. Afamin data were available in 3,059 KORA F4 participants. The second follow-up (KORA FF4) was conducted in 2013/2014 and 2,161 former F4 participants took part. Of them, 2,148 had data on afamin. Prevalent type 2 diabetes at KORA F4 was defined as self-reported and validated by hospital records or by questioning the responsible physician, or as current use of antidiabetic medication. Additionally, a validation of the diabetes type was requested. If no type validation, but also no contradicting information was given, diabetic participants were assumed to have type 2 diabetes. In the type 2 diabetes incidence analyses, only those participants who attended both the KORA F4 and KORA FF4 studies were included. The percentage of loss-to-follow up could be quantified with 30%. Incident type 2 diabetes in KORA FF4 was assessed and defined as specified for prevalent type 2 diabetes in KORA F4.

All KORA F4 participants without known diabetes were to receive a standard oral glucose tolerance test (OGTT), carried out in the morning (7:00 am to 11:00 am). Participants were asked to fast for 10h overnight, to avoid heavy physical activity on the day before examination and to refrain from smoking before and during the test. Exclusion criteria for the OGTT were: (i) consumption of foods or drinks containing calories within 8h before the fasting blood draw; (ii) medical contraindications such as gastrointestinal disease, fructose-intolerance, currant allergy, weakness, risk of hypoglycaemia, or pregnancy. Fasting venous blood was sampled for glucose determination and 75g of anhydrous glucose given (Dextro OGT, Boehringer Mannheim, Germany, containing currant extract). In order to keep type 2 diabetes definitions comparable across the investigated study populations, KORA F4 OGTT data were not used for type 2 diabetes definition in the current pooled study but for prediabetes definition and for calculation of the whole-body insulin sensitivity index ISI(composite) as well as risk discrimination and reclassification analyses that were done in KORA F4 only.

Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or antihypertensive drug treatment in case the individual was aware of the disease.

In both cohorts, the cholesterol-esterase method (CHOL Flex, Dade-Behring, Germany) was applied to determine total cholesterol. For triglyceride and HDL cholesterol concentrations the TGL Flex and AHDL Flex method (Dade-Behring) and for LDL cholesterol a direct method (ALDL, Dade-Behring) was used, respectively. In KORA F4, fasting serum insulin was assessed by ELISA (Invitrogen, Darmstadt, Germany) and fasting serum glucose

using a hexokinase method (GLU Flex, Dade Behring, Deerfield, IL). The following formula was applied to calculate HOMA-IR: fasting insulin [µU/mL] * fasting glucose [mg/dL] / 405 [(2)]. The quantification of HbA1c was done in hemolyzed whole blood in KORA F4 with a cation-exchange HPLC photometric assay on an Adams HA-8160 Hemoglobin Analysis System (Arkray Inc., distributed by A. Menarini Diagnostics, Florence, Italy) and in KORA F3 with a turbidimetric immunoassay method (Tina-quant® Hämoglobin A1c) on a Dimension RXL instrument, Dade-Behring Inc., Newark U.S.A. High-sensitivity CRP (hs-CRP) was measured by immunonephelometry on a BN II analyzer using the CardioPhase assay from Siemens (Marburg, Germany) [(3,4)]

CoLaus Study

The CoLaus (Cohorte Lausannoise) Study was designed to examine the epidemiology and genetic determinants of cardiovascular disease. In total, 6,188 Caucasian participants, 3,251 females and 2,937 males aged between 35 and 75 years, were recruited using a **simple non-stratified random sample of the population registry of the city of Lausanne**, Switzerland [(5)]. The participation rate was 41% and all participants came to the outpatient clinic of the University Hospital of Lausanne in the morning after an overnight fast. Venous blood samples were drawn and routine clinical assays were performed at the Clinical Laboratory of the Centre Hospitalier Universitaire Vaudois (CHUV). Total cholesterol was measured by CHOD-PAP, HDL cholesterol by CHOD-PAP + PEG + cyclodextrin and triglycerides by GPO-PAP. LDL cholesterol was calculated based on the Friedewald formula only if triglycerides were <4.6 mmol/l. The measurement of high sensitive CRP (hsCRP) was carried out with a latex– enhanced HS immunoassay (Roche Diagnostics, CH). A solid-phase, two-site chemiluminescent immunometric assay by Diagnostic Products Corporation, Los Angeles, USA was applied for insulin and glucose dehydrogenase (Roche Diagnostics, CH) for glucose measurement. HOMA-IR was estimated as fasting serum insulin (mU/l) * fasting plasma glucose (mmol/l) / 22·5. Hba1c was not available. Afamin was measured in 4,773 participants. In CoLaus, type 2 diabetes was defined as fasting plasma glucose ≥7.0 mmol/L and/or oral hypoglycaemic or insulin treatment. In case of diabetes without self-reported type 1 diabetes, a participant was defined to have type 2 diabetes.

Cardiovascular Risk in Young Finns Study (YFS)

The YFS is a **prospective multicenter study** from Finland initiated in 1980 (n=3,596, baseline age range 3–18 years) with several follow-ups over a time period of 30 years. Main aim is the investigation of risk factors for cardiometabolic outcomes [(6,7)].

Detailed data were collected by questionnaires, physical measurements, and blood tests, including information on general health status, serum lipids, insulin, obesity indices, blood pressure, and smoking status. In addition, risk factors such as C-reactive protein (CRP) have been measured. After an overnight fast venous blood samples were drawn and stored at −70°C. Serum triglyceride concentration was measured using the enzymatic glycerol kinase–glycerol phosphate oxidase method (Triglyceride reagent, Beckman Coulter Biomedical, Ireland). Serum total cholesterol, HDL cholesterol (after precipitation of low density lipoprotein (LDL) and very low density lipoprotein levels were assessed with dextran sulfate–Mg2+ by the enzymatic cholesterol esterase–cholesterol oxidase method (Cholesterol reagent, Beckman Coulter Biomedical). An enzymatic hexokinase method (Glucose reagent, Beckman Coulter Biomedical) was applied to measure serum glucose concentrations. Serum insulin concentration was examined by microparticle enzyme immunoassay kit (Abbott Laboratories, Chicago, IL) [(8)]. LDL-cholesterol was determined by the Friedewald formula in participants with triglyceride concentrations <4.0 mmol/l. Afamin values are available from 2,270 individuals in the 2001 follow-up which served as our baseline investigation. The data for incident type 2 diabetes are taken from the 2007 or the 2011 follow-up investigations. Of included participants at baseline, 13% were lost to follow-up. Glycated hemoglobin A1c (HbA1c) was not yet available in 2001. Insulin resistance was estimated based on the HOMA index, i.e. the product of fasting glucose and insulin divided by the constant 22.5. The diagnosis of type 2 diabetes was based on fasting glucose concentrations ≥7mmol/l or HbA1c ≥6.5% or self-reported diabetes or use of medication [(9)].

NHLBI Family Heart Study (FamHS)

The Family Heart Study was initiated in 1992 with the ascertainment of 1,200 families with approximately 6,000 individuals, half randomly sampled, and half selected because of an excess of coronary heart disease (CHD) or risk factor abnormalities and funded by the National Heart, Lung, and Blood Institute (NHLBI) [(10)]. The FamHS is a **prospective study** that investigates the genetic and non-genetic determinates of atherosclerosis. Study participants belonging to the largest pedigrees were invited for a second clinical examination in 2002/03.

Fasting triglyceride concentrations were assayed using triglyceride GB reagent and serum total cholesterol using a commercial cholesterol oxidase method on the Roche COBAS FARA centrifugal analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula in

case of triglyceride concentrations <4.5 mmol/L (400 mg/dL). Otherwise, LDL was measured by ultracentrifugation [(11)]. Fasting glucose was examined by a thin film adaptation of an enzymatic glucose-oxidase spectrophotometric procedure using the Vitros analyzer (Ortho Clinical Diagnostics, Rochester, NY) and insulin concentrations by the coated-tube radioimmunoassay method (Diagnostic Products Corporation, Los Angeles, CA) [(12)]. Type 2 diabetes was defined as intake of hypoglycaemic agents, participants reporting a previous clinical diagnosis of type 2 diabetes, or fasting glucose at or above 7 mmol/L. Individuals with type 1 diabetes and age of type 2 diabetes diagnosed before an age of 20 years were excluded. In the current analysis, 1,877 participants of Caucasian origin with available afamin values were included. Finally, 36% of participants were lost to follow-up.

Bruneck-Study

The **prospective, population-based** Bruneck Study was designed to investigate the epidemiology and pathogenesis of atherosclerosis [(13,14)]. In 1990, a random sample including 1,000 subjects of Caucasian origin recruited from the entire population of Bruneck was stratified according to sex and age with 125 subjects of each sex and 5th to 8th decade of age. The participation rate was 93.6% resulting in 919 subjects with complete data. In an interval of five years, follow-up examinations were performed. The baseline for this investigation was the 1995 examination and follow-up data were taken from the 2010 investigation. Of the 826 subjects included at baseline, all had afamin data and detailed information on prevalent and incident diabetes available. All laboratory measurements were determined in samples collected in 1995 and measured by validated standard laboratory methods as described previously [(14,15)]. HbA1c was determined by high performance liquid chromatography (DCCT-aligned assay and insulin resistance by homeostasis model assessment (HOMA-IR) applying the formula fasting plasma glucose in mmol/l × fasting serum insulin in mU/l divided by 22.5. Definition of type 2 diabetes was based on the 1997 American Diabetes Association criteria (fasting glucose ≥126 mg/dL, i.e. ≥7 mmol/L) and/or receiving anti-diabetic treatment and diabetes diagnosis validated through medical records [(16)].

SAPHIR-Study

The SAPHIR Study (Salzburg Atherosclerosis Prevention Program in subjects at High Individual Risk) is an **observational study** accomplished in the years 1999 to 2002 based on 1,770 **healthy unrelated Caucasian subjects**. The recruitment of study participants was

done through health screening programs in large companies in and around the city of Salzburg [17]. Clincial examinations were performed with a main focus on CVD risk factors and lipid metabolism. After an overnight fasting period, venous EDTA blood was collected. Plasma was gathered by low-speed centrifugation and stored at −70°C. Afamin was available in 1,499 participants at the baseline examination. Follow-up examinations were conducted between 2002 and 2008 with a mean follow-up time of 4.59 years; range: 2.10-8.42 years, 22% loss to follow-up. Type 2 diabetes was defined according to the 1997 American Diabetes Association criteria (fasting glucose ≥126 mg/dL) and/or receiving anti-diabetic treatment and diabetes diagnosis validated through medical records [17].

Second Northwick Park Heart Study (NPHS-II)

The **prospective** Second Northwick Park Heart Study (NPHS-II) included 3,052 **unrelated healthy middle-aged men from nine general practices** in the United Kingdom [18]. Baseline characteristics were obtained by questionnaire completed at study entry in 1989. Of the initial cohort, 3,012 men were Caucasian and 2,674 eligible men had afamin measured. These men were prospectively followed with the aim to comprehensively study CVD risk factors and outcomes. Only 3% of participants could not be included at follow-up. For all examinations, participants were non-fasting, but have avoided smoking, vigorous exercise or heavy meals from midnight the day before. Data on lifestyle habits, anthropometrics, blood pressure and various blood biomarkers were collected at the baseline and prospective follow-up investigations. Lipids, total cholesterol, and triglyceride concentrations were gathered with automated enzyme procedures. More details on recruitment and measurements have been reported elsewhere [19]. Prevalent diabetes was defined by self-report (answer to the question: have you ever had diabetes?) in the Second Northwick Park Heart Study (NPHS-II) and diagnosis of incident diabetes was validated through medical records (from a note search undertaken in 2005).

Measures of insulin resistance

Besides the homeostasis model assessment-estimated insulin resistance (HOMA-IR) we calculated the whole-body insulin sensitivity index (ISI(composite)) [20], a valid surrogate measure of data derived from euglycemic insulin clamp, based on the formula: ISI = 10,000 / sqrt ((fasting glucose (mg/dL) * fasting insulin ((µlU/ml))*(2-h glucose (mg/dL) * 2-h insulin (µlU/ml))) as recently applied in KORA F4.

HOMA-IR and whole-body ISI(composite) were also analysed divided by a cut-off of 2.5. Whole-body IS (composite) values ≥2.5 reflect insulin sensitivity, values <2.5 insulin

resistance [21]. For HOMA-IR values ≥2.5 refer to insulin resistance, and values <2.5 to insulin sensitivity. Data on whole-body ISI(composite) were only available in individuals ≥ 62 years of age [22].

Measurement of afamin plasma concentrations

As previously described [23,24] afamin was quantified with a custom-made double-antibody sandwich ELISA using an affinity-purified biotinylated polyclonal anti-afamin 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 that was initially calibrated with a primary standard served as the assay standard. Afamin purified to homogeneity from human plasma was originally used as the primary standard and the protein concentration of this standard was estimated by quantitative amino-acid compositional analysis. Within-run and between-run coefficients of variation were 3.3% and 6.2%, respectively (mean concentration 73 mg/L) [25]. The four same control samples were added to each assay plate using new aliquots each time which were thawed the first time. These control samples were used in all eight studies and were assayed in duplicates. These four samples were monitored throughout the entire project and the assay was repeated when more than one control samples showed a divergent result of more than 10% from the expected values. The intra-assay coefficient of variation (CV) was calculated from the mean and standard deviation (SD) of each of the measured four control samples using the formula CV (%) = SD * 100 / mean using 284 duplicate measurements. The inter-assay CV was calculated using the same formula using the values of the same four controls samples included in 71 runs over a period of six months. The samples of all study participants for each study were measured in a random way independent of a case-control status and the lab personnel was blinded to all variables except the study name. Afamin concentrations were measured for all studies in the laboratory at the Medical University of Innsbruck. A previous report on the assay evaluation described afamin as a robust, stable analyte that is virtually unchanged under different storage conditions. It is independent of sex, fasting state, and a daily and monthly rhythm [25]. In this pooled analysis, data on afamin concentrations was available in 20,136 individuals.

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Supplementary Table 1: Clinical and laboratory data of participants with available afamin measurements in the Bruneck Study (n=826), KORA F3 Study (n=3,158), KORA F4 Study (n=3,059), SAPHIR Study (n=1,499), CoLaus Study (n=4,773), NPHS-II Study (n=2,674), YFS Study (n=2,270), and FamHS Study (n=1,877).

	Study population			
	Bruneck (n=826)	**KORA F3* (n=3,158)**	**KORA F4 (n=3,059)**	**SAPHIR (n=1,499)**
Age, yrs (minimum-maximum)	63±11 53/63/72 (45-85)	57±13 46/57/67 (35-84)	56±13 44/56/67 (32-81)	51±6 46/52/55 (39-67)
Gender: male/female: n, %	414/412 50.1/49.9	1533/1625 48.5/51.5	1477/1582 48.3/51.7	1010/489 67.4/32.6
Smoking (Non-smoker/Ex-smoker/Smoker): n, %	452/213/161 54.7/25.8/19.5	1325/1101/551 44.5/37.0/18.5	1349/1160/546 44.2/38.0/17.9	957/214/328 63.8/14.3/21.9
Follow-up time (years)	12.5 ± 4.3 10.3/15.0/15.0	4.5 ± 0.4 4.2/4.5/4.8	6.5 ± 0.3 6.3/6.4/6.6	4.6± 0.7 4.3/4.4/4.6
Afamin (mg/L)	62.6±15.3 52.1/61.5/71.7	71.4±17.1 59.3/69.7/81.4	70.6±17.2 58.8/68.7/80.6	66.2±14.3 56.4/64.1/73.9
Body mass index, kg/m²	25.7±3.9	27.6±4.6	27.6±4.8	26.8±4.1
Obesity (BMI ≥30: n, (%))	115 (13.9)	842 (26.9)	809 (26.6)	280 (18.7)
Systolic blood pressure (mmHg)	148±21	131±20	122±18	138±18
Diastolic blood pressure (mmHg)	87±9	82±11	75±10	86±12
Hypertension: n, %	564 (68)	1576 (50)	1169 (38)	821 (55)
Antihypertensive medication: n, %	230 (28)	996 (32)	944 (31)	212 (14)
Waist circumference (cm)	90±11	95±13	94±14	95±12
Total cholesterol, mg/dL	230±42	218±40	216±40	227±39
HDL cholesterol, mg/dL	59±16	59±17	56±14	59±16
LDL cholesterol, mg/dL	145±38	128±33	136±35	145±36
Triglycerides, mg/dL	132±81 81/111/158	165±126 88/135/201	125±89 72/104/151	126±89 72/101/151
Use of lipid lowering drugs: n, %	38 (4.6)	337 (10.7)	382 (12.5)	63 (4.2)
Type 2 Diabetes: n, %	93 (11.2)	260 (8.3)	245 (8.0)	41 (2.7)
Diabetes medication: n, %	38 (4.6)	205 (6.5)	179 (5.9)	23 (1.5)
HbA1c (%)†	5.5±0.7 5.1/5.4/5.8	5.4±0.5 5.1/5.3/5.5	5.6±0.6 5.2/5.5/5.7	5.6±0.6 5.4/5.6/5.7
HOMA-IR	4.0±5.3 2.1/3.0/4.3	NA	2.1±8.3 0.6/1.0/1.7	1.8±1.5 0.9/1.4/2.1
Fasting glucose (mg/dL)	102±24 91/97/107	NA	98±19 88/94/102	93±18 85/91/98
Fasting insulin (µlU/ml)	15±13 9/12/17	NA	9±34 3/4/7	7±5 4/6/9
eGFR (mL/min/1.73m²)	79±15	83±18	84±17	95±12
Hs-CRP (mg/L)	3.4±7.4 1.0/2.0/3.0	NA	2.5±5.3 0.6/1.2/2.6	2.8±6.6 0.8/1.5/2.9

Values are provided as mean and standard deviation and 25th, 50th and 75th percentile where appropriate and in case of non-normal distribution as not indicated otherwise or number, % (=valid percent considering missing values). To convert mg/dL in mmol/L multiply by 0.0555 for glucose, 0.0259 for cholesterol and 0.0113 for triglycerides. To convert µlU/ml in pmol/L for insulin, multiply by 7.175. * Participants (92.3%) non-fasting; † To convert % to mmol/mol the following formula is used: New (mmol/mol) = 10.93xOld (%) - 23.5 mmol/mol. Hypertension defined according to the JNC7 Criteria (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, and/or receiving antihypertensive treatment); Lipid lowering drugs includes statin and/or fibrate use; Glomerular filtration rate (eGFR) measured according to the CKD-EPI equation [26].

Supplementary Table 1 (continuation): Clinical and laboratory data of participants in those with afamin measurements available in the Bruneck Study (n=826), KORA F3 Study (n=3,158), KORA F4 Study (n=3,059), SAPHIR Study (n=1,499), CoLaus Study (n=4,773), NPHS-II Study (n=2,674), YFS Study (n=2,270), and FamHS Study (n=1,877).

	Study population			
	CoLaus (n=4,773)	**NPHS-II* (n=2,674)**	**YFS (n=2,270)**	**FamHS (n=1,877)**
Age, yrs (minimum-maximum)	58±10 49/57/66 (40–82)	59±3 56/59/62 (50-66)	32±5 27/33/36 (24-39)	52±14 39/53/63 (25-89)
Gender: male/female: n, %	2235/2538 46.8/53.2	2674 (100)	1020/1250 (45/55)	863/1014 46/54
Smoking (Non-smoker/Ex-smoker/Smoker): n, %	1948/1785/1040 40.8/37.4/ 21.8	860/1072/742 32.2/40.1/27.8	1031/402/776 46.7/18.2/35.1	910/492/248 48.5/26.2/13.2
Follow-up time (years)	5.0±0.5 5.0/5.2/5.3	9.2±2.9 8.3/10.0/10.8	9.4±1.4 10.0/10.0/10.0	7.3±0.8 6.7/7.3/7.9
Afamin (mg/L)	73.1±16.6 61.3/71.3/82.8	67.0±15.8 55.9/65.4/76.3	61.4 ± 15.4 50.7/59.0/70.2	65.4±16.3 53.8/63.8/75.3
Body mass index, kg/m²	26.2±4.6	26.6±3.6	25.1±4.4	27.7±5.3
Obesity (BMI ≥30: n, (%)	816 (17.1)	418 (15.8)	276 (12.3)	462 (24.6)
Systolic blood pressure (mmHg)	126±18	134±18	117±13	117±18
Diastolic blood pressure (mmHg)	78±11	82±11	71±11	69±10
Hypertension: n, %	1969 (41.3)	1119 (41.9)	882 (39.3)	557 (29.7)
Antihypertensive medication: n, %	1292 (27.1)	232 (8.7)	51 (2.5)	455 (24.2)
Waist circumference (cm)	92±13	NA	84±12	97±15
Total cholesterol, mg/dL	220±40.1	218±38	200±38	205±39
HDL cholesterol, mg/dL	63±18	66±23	50±12	50±15
LDL cholesterol, mg/dL	133±36	119±39	127±33	125±34
Triglycerides, mg/dL	120±78 71/97/142	186±115 112/155/229	119±76 71/97/142	150±105 84/125/185
Use of lipid lowering drugs: n, %	877 (18.4)	NA	7 (0.3)	163 (8.7)
Type 2 Diabetes: n, %	503 (10.5)	68 (2.5)	17 (0.8)	171 (9.1)
Diabetes medication: n, %	261 (5.5)	26 (1.0)	0 (0)	84 (4.5)
HbA1c (%)†	NA	NA	NA	NA
HOMA-IR	2.5±7.1 1.1/1.6/2.7	NA	1.8±1.5 1.0/1.4/2.1	3.3±7.6 1.4/2.1/3.3
Fasting glucose (mg/dL)	106±20 95/103/110	NA	90±9 85/90/95	100±28 88/94/102
Fasting insulin (µlU/ml)	9±16 4/7/10	NA	8±6 5/6/9	12±15 6/9/14
eGFR (mL/min/1.73m²)	83±15	75±10	114± 6	84±16
Hs-CRP (mg/L)	2.5±3.6 0.7/1.3/2.8	3.9±3.9 1.2/3.5/5.1	1.9±3.9 0.3/0.8/1.9	NA

Values are provided as mean and standard deviation and 25th, 50th and 75th percentile where appropriate and in case of non-normal distribution as not indicated otherwise or number, % (=valid percent considering missing values). To convert mg/dL in mmol/L multiply by 0.0555 for glucose, 0.0259 for cholesterol and 0.0113 for triglycerides. To convert µlU/ml in pmol/L for insulin, multiply by 7.175. * The NPHS-II Study includes only males. † To convert % to mmol/mol the following formula is used: New (mmol/mol) = 10.93xOld (%) - 23.5 mmol/mol. Hypertension defined according to the JNC7 Criteria (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, and/or receiving antihypertensive treatment); Lipid lowering drugs includes statin and/or fibrate use; Glomerular filtration rate (eGFR) measured according to the CKD EPI equation [(26)].

Supplementary Table 2: I^2 index and p value from chi-square based Q-statistic based on an age- and sex-adjusted model

Outcome	All cohorts		Excluding KORA F3 and NPHS-II	
	I^2 index	p value (Q-statistic)	I^2 index	p value (Q-statistic)
Prevalent type 2 diabetes				
Afamin on a continuous scale	63.50	0.008	14.25	0.32
Afamin categorized by quartiles				
Afamin 2nd vs. 1st quartile	34.86	0.16	34.12	0.19
Afamin 3rd vs. 1st quartile	21.62	0.26	0 *	0.50
Afamin 4th vs. 1st quartile	50.13	0.06	0 *	0.41
Incident type 2 diabetes				
Afamin on a continuous scale	0 *	0.55	0 *	0.47
Afamin categorized by quartiles				
Afamin 2nd vs. 1st quartile	35.30	0.15	6.71	0.38
Afamin 3rd vs. 1st quartile	0 *	0.65	0 *	0.50
Afamin 4th vs. 1st quartile	13.89	0.32	21.05	0.28
Continuous type 2 diabetes-related phenotypes				
Ln-HbA1c (%) †	78.36	0.003	NA	NA
Ln-Glucose (mg/dL) ‡	95.21	<0.0001	NA	NA
Ln-Insulin (µlU/ml) ‡	93.57	<0.0001	NA	NA
Ln-HOMA Index ‡	94.43	<0.0001	NA	NA

Ln refers to log-transformation based on the natural logarithm (ln).

* In case of $I^2 = 0$, the random effects model equals the fixed effects model

† Cohorts included: Bruneck Study, SAPHIR Study, KORA F3 and KORA F4 Study (those without type 2 diabetes diagnosis at baseline). To convert % to mmol/mol the following formula is used: New (mmol/mol) = 10.93xOld (%) - 23.5 mmol/mol.

‡ Includes all cohorts except KORA F3 and NPHS-II (those without type 2 diabetes diagnosis at baseline)

NA, not applicable

Supplementary Table 3: Logistic regression analysis of afamin (increment 10 mg/L) on prevalent and incident type 2 diabetes

Prevalent Type 2 Diabetes (total number): yes = 1,398, no = 18,696					
Study	**Type 2 Diabetes (1 = yes / 0 = no (=ref.)** [*]	**OR (95% CI)** [†]	**P**	**OR (95% CI)** [‡]	**P**
Bruneck Study	(1 = 93 / 0 = 733)	1.68 (1.45-1.95)	$8.47*10^{-12}$	1.42 (1.19-1.68)	$7.26*10^{-5}$
SAPHIR Study	(1 = 41 / 0 = 1,447)	1.54 (1.28-1.84)	$2.51*10^{-6}$	1.25 (1.01-1.55)	0.043
FamHS Study	(1 = 171 / 0 = 1,706)	1.38 (1.25-1.52)	$9.17*10^{-11}$	1.19 (1.06-1.34)	$3.70*10^{-3}$
YFS Study	(1 =17 / 0 = 2,253)	1.41 (1.11-1.78)	$4.42*10^{-3}$	1.18 (0.86-1.62)	0.293
NPHS-II Study	(1 = 68 / 0 = 2,606)	1.26 (1.10-1.44)	$7.19*10^{-4}$	1.06 (0.88-1.28)	0.535
KORA F4 Study	(1 = 245 / 0 = 2,805)	1.40 (1.30-1.51)	$2.60*10^{-19}$	1.23 (1.13-1.34)	$2.18*10^{-6}$
KORA F3 Study	(1 = 260 / 0 = 2,876)	1.25 (1.16-1.34)	$2.24*10^{-9}$	1.05 (0.96-1.15)	0.260
CoLaus Study	(1 = 503 / 0 = 4,270)	1.44 (1.36-1.52)	$3.05*10^{-38}$	1.21 (1.14-1.29)	$1.28*10^{-9}$
Meta-analysis including all studies [§]		**1.40 (1.31-1.48)**	**$2.54*10^{-27}$**	**1.19 (1.12-1.26)**	**$5.96*10^{-08}$**
Meta-analysis excluding KORA F3 & NPHS-II [‖]		**1.44 (1.38-1.50)**	**$5.64*10^{-61}$**	**1.23 (1.17-1.28)**	**$2.62*10^{-20}$**
Incident Type 2 Diabetes (total number): yes = 585, no = 12,762					
Study	**Type 2 Diabetes (1 = yes / 0 = no (=ref.)** [*]	**OR (95% CI)** [†]	**P**	**OR (95% CI)** [‡]	**P**
Bruneck Study	(1 = 52 / 0 = 681)	1.48 (1.21-1.82)	$1.28*10^{-4}$	1.32 (1.04-1.68)	0.025
SAPHIR Study	(1 = 78 / 0 = 1,087)	1.64 (1.41-1.92)	$2.28*10^{-10}$	1.33 (1.11-1.59)	$1.83*10^{-3}$
FamHS Study	(1 = 83 / 0 = 1,036)	1.47 (1.30-1.66)	$1.54*10^{-9}$	1.37 (1.18-1.59)	$3.54*10^{-5}$
YFS Study	(1 = 55 / 0 = 1,900)	1.54 (1.35-1.76)	$1.84*10^{-10}$	1.35 (1.13-1.61)	$9.55*10^{-4}$
NPHS-II Study	(1 = 135 / 0 = 2,391)	1.42 (1.29-1.57)	$1.34*10^{-12}$	1.18 (1.04-1.35)	0.013
KORA F4 Study	(1 = 86 / 0 = 1,925)	1.60 (1.42-1.80)	$7.67*10^{-15}$	1.40 (1.23-1.60)	$5.49*10^{-7}$
KORA F3 Study	(1 = 52 / 0 = 2,459)	1.46 (1.27-1.68)	$9.64*10^{-8}$	1.28 (1.09-1.50)	$2.70*10^{-3}$
CoLaus Study	(1 = 44 / 0 = 1,283)	1.32 (1.12-1.57)	$1.19*10^{-3}$	1.17 (0.96-1.42)	0.124
Meta-analysis including all studies [§]		**1.49 (1.42-1.56)**	**$5.97*10^{-62}$**	**1.30 (1.23-1.38)**	**$3.53*10^{-19}$**
Meta-analysis excluding KORA F3 & NPHS-II [‖]		**1.52 (1.43-1.61)**	**$4.52*10^{-45}$**	**1.34 (1.25-1.43)**	**$1.90*10^{-16}$**

[*] Numbers refer to the age- and sex-adjusted model

[†] Adjusted for age and sex;

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

[§] Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model

[‖] Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model without KORA F3 and NPHS-II. These two studies did not ask participants to be fasting at their examination.

Supplementary Table 4: Logistic regression analysis of afamin (divided into quartiles) on prevalent type 2 diabetes

Study [*]	Type 2 Diabetes (1 = yes / 0 = no (=ref.) [†]	OR (95% CI) [‡]	P	OR (95% CI) [§]	P
Bruneck Study	1st quartile (1 = 12 / 0 = 194)	Reference			
Bruneck Study	2nd quartile (1 = 13 / 0 = 195)	1.19 (0.52-2.72)	0.684	1.07 (0.46-2.52)	0.869
Bruneck Study	3rd quartile (1 = 23 / 0 = 183)	2.31 (1.09-4.88)	0.029	1.41 (0.62-3.19)	0.407
Bruneck Study	4th quartile (1 = 45 / 0 = 161)	5.29 (2.65-10.58)	$2.42*10^{-6}$	2.25 (1.02-5.00)	0.045
SAPHIR Study	1st quartile (1 = 4 / 0 = 371)	Reference			
SAPHIR Study	2nd quartile (1 = 3 / 0 = 369)	0.70 (0.15-3.16)	0.642	0.50 (0.11-2.33)	0.375
SAPHIR Study	3rd quartile (1 = 8 / 0 = 364)	1.71 (0.51-5.76)	0.388	0.98 (0.27-3.47)	0.970
SAPHIR Study	4th quartile (1 = 26 / 0 = 343)	6.01 (2.06-17.50)	$1.01*10^{-3}$	1.96 (0.60-6.41)	0.266
FamHS Study	1st quartile (1 = 22 / 0 = 447)	Reference			
FamHS Study	2nd quartile (1 = 22 / 0 = 447)	0.74 (0.39-1.41)	0.355	0.45 (0.22-0.90)	0.023
FamHS Study	3rd quartile (1 = 42 / 0 = 427)	1.38 (0.81-2.37)	0.238	0.87 (0.46-1.64)	0.670
FamHS Study	4th quartile (1 = 85 / 0 = 385)	3.11 (1.93-5.01)	$3.16*10^{-6}$	1.37 (0.75-2.50)	0.310
NPHS-II Study	1st quartile (1 = 12 / 0 = 657)	Reference			
NPHS-II Study	2nd quartile (1 = 8 / 0 = 660)	0.66 (0.27-1.62)	0.361	0.40 (0.15-1.08)	0.071
NPHS-II Study	3rd quartile (1 = 14 / 0 = 655)	1.18 (0.54-2.58)	0.674	0.40 (0.15-1.06)	0.066
NPHS-II Study	4th quartile (1 = 34 / 0 = 634)	2.93 (1.50-5.71)	$2.00*10^{-3}$	1.15 (0.51-2.59)	0.727
KORA F4 Study	1st quartile (1 = 25 / 0 = 736)	Reference			
KORA F4 Study	2nd quartile (1 = 34 / 0 = 728)	1.18 (0.69-2.04)	0.541	0.94 (0.53-1.66)	0.832
KORA F4 Study	3rd quartile (1 = 56 / 0 = 707)	1.88 (1.14-3.09)	0.013	1.13 (0.66-1.94)	0.651
KORA F4 Study	4th quartile (1 = 130 / 0 = 634)	4.53 (2.87-7.15)	$9.17*10^{-11}$	2.25 (1.34-3.77)	$2.05*10^{-3}$
KORA F3 Study	1st quartile (1 = 46 / 0 = 739)	Reference			
KORA F3 Study	2nd quartile (1 = 41 / 0 = 745)	0.94 (0.60-1.47)	0.786	0.76 (0.47-1.22)	0.249
KORA F3 Study	3rd quartile (1 = 64 / 0 = 721)	1.32 (0.88-1.98)	0.182	0.78 (0.49-1.21)	0.265
KORA F3 Study	4th quartile (1 = 109 / 0 = 671)	2.57 (1.76-3.74)	$8.71*10^{-7}$	1.07 (0.69-1.68)	0.756
CoLaus Study	1st quartile (1 = 48 / 0 = 1,146)	Reference			
CoLaus Study	2nd quartile (1 = 85 / 0 = 1,108)	1.74 (1.20-2.53)	$3.64*10^{-3}$	1.38 (0.94-2.04)	0.101
CoLaus Study	3rd quartile (1 = 119 / 0 = 1,074)	2.45 (1.71-3.50)	$7.42*10^{-7}$	1.59 (1.09-2.31)	0.016
CoLaus Study	4th quartile (1 = 251 / 0 = 942)	5.45 (3.92-7.58)	$7.50*10^{-24}$	2.46 (1.71-3.52)	$9.58*10^{-7}$
Meta-analysis including all studies [‖]					
	1st quartile (1 = 169 / 0 = 4,290)	Reference			
	2nd quartile (1 = 206 / 0 = 4,252)	**1.09 (0.81-1.46)**	**0.572**	**0.80 (0.56-1.16)**	**0.238**
	3rd quartile (1 = 326 / 0 = 4,131)	**1.74 (1.38-2.20)**	**$3.47*10^{-6}$**	**1.02 (0.74-1.40)**	**0.917**
	4th quartile (1 = 680 / 0 = 3,770)	**3.91 (2.97-5.14)**	**$2.10*10^{-22}$**	**1.72 (1.27-2.33)**	**$5.09*10^{-4}$**
Meta-analysis excluding KORA F3 & NPHS-II [¶]					
	1st quartile (1 = 111 / 0 = 2,894)	Reference			
	2nd quartile (1 = 157 / 0 = 2,847)	**1.21 (0.85-1.72)**	**0.296**	**0.89 (0.56-1.40)**	**0.609**
	3rd quartile (1 = 248 / 0 = 2,755)	**2.03 (1.60-2.57)**	**$4.82*10^{-9}$**	**1.29 (1.00-1.67)**	**0.052**
	4th quartile (1 = 537 / 0 = 2,465)	**4.66 (3.75-5.79)**	**$3.54*10^{-44}$**	**2.15 (1.68-2.74)**	**$1.14*10^{-9}$**

[*] The YFS Study is not included in these analyses due to low numbers of cases.

[†] Numbers refer to the age- and sex-adjusted model

[‡] Adjusted for age and sex

[§] Adjusted for age, sex, HDL cholesterol, triglycerides, body mass index and hypertension

[‖] Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model

[¶] Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model without KORA F3 and NPHS-II. These two studies did not ask participants to be fasting at their examination.

Supplementary Table 5: Logistic regression analysis of afamin (divided into quartiles) on incident type 2 diabetes

Study	Type 2 Diabetes (1=yes / 0=no (=ref.) *	OR (95% CI) †	P	OR (95% CI) ‡	P
Bruneck Study	1st quartile (1 = 6 / 0 = 188)	Reference			
Bruneck Study	2nd quartile (1 = 10 / 0 = 185)	1.69 (0.60-4.76)	0.320	1.55 (0.54-4.40)	0.412
Bruneck Study	3rd quartile (1 = 14 / 0 = 169)	2.59 (0.97-6.91)	0.057	1.96 (0.70-5.49)	0.202
Bruneck Study	4th quartile (1 = 22 / 0 = 139)	4.96 (1.96-12.57)	0.001	3.14 (1.11-8.89)	0.031
SAPHIR Study	1st quartile (1 = 10 / 0 = 297)	Reference			
SAPHIR Study	2nd quartile (1 = 10 / 0 = 285)	0.97 (0.40-2.37)	0.942	0.61 (0.24-1.54)	0.294
SAPHIR Study	3rd quartile (1 = 17 / 0 = 282)	1.62 (0.72-3.62)	0.240	0.69 (0.29-1.64)	0.396
SAPHIR Study	4th quartile (1 = 41 / 0 = 223)	5.05 (2.46-10.35)	$9.71*10^{-6}$	1.61 (0.72-3.61)	0.250
FamHS Study	1st quartile (1 = 9 / 0 = 280)	Reference			
FamHS Study	2nd quartile (1 = 13 / 0 = 269)	1.28 (0.53-3.10)	0.591	1.03 (0.42-2.50)	0.955
FamHS Study	3rd quartile (1 = 23 / 0 = 268)	2.13 (0.98-4.64)	0.056	1.46 (0.63-3.40)	0.383
FamHS Study	4th quartile (1 = 38 / 0 = 219)	4.08 (1.93-8.63)	$2.00*10^{-4}$	2.49 (1.08-5.79)	0.033
YFS Study	1st quartile (1 = 4 / 0 = 486)	Reference			
YFS Study	2nd quartile (1 = 10 / 0 = 478)	2.69 (0.84-8.65)	0.097	2.10 (0.64-6.90)	0.220
YFS Study	3rd quartile (1 = 9 / 0 = 479)	2.44 (0.74-7.99)	0.141	1.39 (0.41-4.75)	0.600
YFS Study	4th quartile (1 = 32 / 0 = 457)	8.89 (3.11-25.44)	$4.57*10^{-5}$	2.84 (0.87-9.22)	0.083
NPHS-II Study	1st quartile (1 = 17 / 0 = 611)	Reference			
NPHS-II Study	2nd quartile (1 = 10 / 0 = 634)	0.57 (0.26-1.25)	0.157	0.42 (0.18-0.96)	0.041
NPHS-II Study	3rd quartile (1 = 44 / 0 = 593)	2.68 (1.51-4.74)	$7.18*10^{-4}$	1.24 (0.65-2.38)	0.509
NPHS-II Study	4th quartile (1 = 64 / 0 = 553)	4.15 (2.40-7.18)	$3.40*10^{-7}$	1.46 (0.77-2.78)	0.250
KORA F4 Study	1st quartile (1 = 3 / 0 = 545)	Reference			
KORA F4 Study	2nd quartile (1 = 11 / 0 = 507)	3.45 (0.95-12.48)	0.059	2.78 (0.76-10.16)	0.122
KORA F4 Study	3rd quartile (1 = 24 / 0 = 487)	7.40 (2.21-24.84)	$1.12*10^{-3}$	4.72 (1.37-16.29)	0.014
KORA F4 Study	4th quartile (1 = 48 / 0 = 386)	17.05 (5.25-55.37)	$2.37*10^{-6}$	8.31 (2.44-28.28)	$7.09*10^{-4}$
KORA F3 Study	1st quartile (1 = 3 / 0 = 654)	Reference			
KORA F3 Study	2nd quartile (1 = 8 / 0 = 648)	2.66 (0.70-10.08)	0.150	1.70 (0.43-6.72)	0.447
KORA F3 Study	3rd quartile (1 = 13 / 0 = 608)	4.20 (1.19-14.85)	0.026	2.44 (0.67-8.82)	0.176
KORA F3 Study	4th quartile (1 = 28 / 0 = 549)	9.95 (3.00-32.99)	$1.71*10^{-4}$	4.01 (1.13-14.28)	0.032
CoLaus Study	1st quartile (1 = 7 / 0 = 352)	Reference			
CoLaus Study	2nd quartile (1 = 5 / 0 = 376)	0.69 (0.22-2.19)	0.526	0.52 (0.16-1.68)	0.273
CoLaus Study	3rd quartile (1 = 14 / 0 = 296)	2.29 (0.91-5.78)	0.079	1.54 (0.58-4.07)	0.381
CoLaus Study	4th quartile (1 = 18 / 0 = 259)	3.27 (1.34-7.99)	0.009	1.69 (0.63-4.52)	0.295
Meta-analysis including all studies §					
	1st quartile (1 = 59 / 0 = 3,413)	Reference			
	2nd quartile (1 = 77 / 0 = 3,382)	**1.31 (0.83-2.06)**	**0.251**	**1.00 (0.62-1.62)**	**0.996**
	3rd quartile (1 = 158 / 0 = 3,182)	**2.56 (1.88-3.49)**	$\mathbf{2.25*10^{-9}}$	**1.47 (1.04-2.08)**	**0.030**
	4th quartile (1 = 291 / 0 = 2,785)	**5.28 (3.83-7.27)**	$\mathbf{2.64*10^{-24}}$	**2.33 (1.61-3.36)**	$\mathbf{6.66*10^{-6}}$
Meta-analysis excluding KORA F3 & NPHS-II ‖					
	1st quartile (1 = 39 / 0 = 2,148)	Reference			
	2nd quartile (1 = 59 / 0 = 2,100)	**1.43 (0.92-2.22)**	**0.112**	**1.12 (0.68-1.86)**	**0.652**
	3rd quartile (1 = 101 / 0 = 1,981)	**2.40 (1.64-3.52)**	$\mathbf{7.47*10^{-6}}$	**1.51 (0.94-2.44)**	**0.087**
	4th quartile (1 = 199 / 0 = 1,683)	**5.46 (3.63-8.22)**	$\mathbf{4.07*10^{-16}}$	**2.54 (1.66-3.89)**	$\mathbf{9.68*10^{-6}}$

* Numbers refer to the age and sex adjusted model

† Adjusted for age and sex; ‡ Adjusted for age, sex, HDL cholesterol, triglycerides, body mass index and hypertension

§ Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model; ‖ Meta-analysis beta estimate (recalculated to an odds ratio and corresponding 95% CI) and P-values derived from a random effects model without KORA F3 and NPHS-II. These two studies did not ask participants to be fasting at their examination.

Supplementary Table 6: Linear regression analysis 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	
Parameter / Study (n individuals)	**ß (95% CI)** [*]	**P**	**ß (95% CI)** [†]	**P**
Ln-HbA1c				
Bruneck Study (n=733)	0.006 (0.002-0.010)	$4.50*10^{-3}$	0.003 (-0.001-0.008)	0.166
SAPHIR Study (n=1,425)	0.007 (0.004-0.009)	$3.92*10^{-9}$	0.004 (0.002-0.007)	$1.65*10^{-3}$
KORA F3 Study (n=2,869)	0.005 (0.003-0.006)	$3.80*10^{-13}$	0.002 (0.0003-0.003)	0.018
KORA F4 Study (n=2,801)	0.008 (0.007-0.009)	$1.01*10^{-31}$	0.005 (0.003-0.006)	$8.37*10^{-10}$
CoLaus Study	---	---	---	---
NPHS-II Study	---	---	---	---
YFS Study	---	---	---	---
FamHS Study	---	---	---	---
Meta-analysis [‡]	**0.006 (0.004-0.008)**	**$4.41*10^{-10}$**	**0.003 (0.002-0.005)**	**$3.09*10^{-4}$**
Ln-HOMA Index				
Bruneck Study (n=733)	0.144 (0.121-0.168)	$1.46*10^{-33}$	0.082 (0.056-0.109)	$1.11*10^{-9}$
SAPHIR Study (n=1,441)	0.249 (0.231-0.268)	$3.90*10^{-157}$	0.152 (0.134-0.171)	$6.37*10^{-59}$
FamHS Study (n=1,706)	0.171 (0.151-0.190)	$1.37*10^{-64}$	0.104 (0.084-0.124)	$3.40*10^{-25}$
YFS Study (n=2,252)	0.159 (0.145-0.173)	$1.32*10^{-97}$	0.079 (0.064-0.093)	$8.66*10^{-26}$
KORA F4 Study (n=2,751)	0.222 (0.202-0.243)	$1.71*10^{-101}$	0.134 (0.112-0.156)	$7.21*10^{-33}$
CoLaus Study (n=4,270)	0.176 (0.165-0.187)	$8.77*10^{-210}$	0.111 (0.100-0.122)	$1.05*10^{-91}$
KORA F3 Study	---	---	---	---
NPHS-II Study	---	---	---	---
Meta-analysis [‡]	**0.187 (0.158-0.216)**	**$3.00*10^{-36}$**	**0.110 (0.089-0.132)**	**$1.37*10^{-23}$**
Ln-Insulin				
Bruneck Study (n=733)	0.129 (0.106-0.151)	$8.82*10^{-30}$	0.071 (0.046-0.097)	$2.74*10^{-8}$
SAPHIR Study (n=1,441)	0.228 (0.211-0.245)	$2.77*10^{-150}$	0.136 (0.119-0.154)	$1.35*10^{-54}$
KORA F3 Study	---	---	---	---
KORA F4 Study (n=2,754)	0.203 (0.183-0.222)	$3.34*10^{-90}$	0.121 (0.100-0.143)	$9.44*10^{-29}$
CoLaus Study (n=4,270)	0.164 (0.153-0.174)	$2.51*10^{-202}$	0.104 (0.094-0.115)	$2.47*10^{-89}$
NPHS-II Study	---	---	---	---
YFS Study (n=2,252)	0.153 (0.140-0.166)	$1.81*10^{-102}$	0.077 (0.063-0.090)	$1.28*10^{-27}$
FamHS Study (n=1,706)	0.155 (0.136-0.174)	$4.29*10^{-59}$	0.094 (0.075-0.113)	$1.67*10^{-22}$
Meta-analysis [‡]	**0.172 (0.146-0.198)**	**$3.32*10^{-39}$**	**0.101 (0.083-0.120)**	**$1.51*10^{-26}$**
Ln-Glucose				
Bruneck Study (n=733)	0.016 (0.011-0.021)	$7.21*10^{-10}$	0.011 (0.005-0.017)	$3.38*10^{-4}$
SAPHIR Study (n=1,442)	0.022 (0.018-0.025)	$2.39*10^{-31}$	0.016 (0.012-0.020)	$1.84*10^{-13}$
KORA F3 Study	---	---	---	---
KORA F4 Study (n=2,779)	0.020 (0.017-0.022)	$6.04*10^{-68}$	0.012 (0.010-0.015)	$1.01*10^{-21}$
CoLaus Study (n=4,270)	0.012 (0.011-0.014)	$3.08*10^{-49}$	0.007 (0.005-0.009)	$3.73*10^{-14}$
NPHS-II Study	---	---	---	---
YFS Study (n=2,253)	0.006 (0.004-0.008)	$2.64*10^{-07}$	0.002 (-0.0004-0.005)	0.108
FamHS Study (n=1,706)	0.015 (0.012-0.018)	$8.30*10^{-24}$	0.010 (0.006-0.013)	$8.90*10^{-9}$
Meta-analysis [‡]	**0.015 (0.010-0.020)**	**$4.68*10^{-10}$**	**0.009 (0.006-0.013)**	**$7.48*10^{-7}$**

N refer to the age and sex adjusted model

[*] Adjusted for age and sex

[†] Adjusted for age, sex, HDL cholesterol, triglycerides, body mass index and hypertension

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

Supplementary Table 7: Logistic regression analysis of afamin (increment 10 mg/L) on incident type 2 diabetes in 6 out of 8 cohorts additionally including glucose concentrations.

Study	Type 2 Diabetes (1 = yes / 0 = no (=ref.)	OR (95% CI) *	P	OR (95% CI) †	P
Bruneck Study	(1=52 / 0=681)	1.26 (0.98-1.62)	0.069	1.21 (0.94-1.56)	0.144
YFS Study	(1=55 / 0=1,837)	1.34 (1.11-1.61)	0.002	1.31 (1.08-1.58)	0.005
FamHS Study	(1=79 / 0=978)	1.28 (0.09-1.50)	0.002	1.21 (1.03-1.42)	0.021
KORA F4 Study	(1=71 / 0=1,900)	1.35 (1.17-1.57)	$6.19*10^{-5}$	1.27 (1.09-1.49)	0.003
CoLaus Study	(1=44 / 0=1,283)	1.15 (0.94-1.40)	0.166	1.07 (0.87-1.31)	0.531
SAPHIR Study	(1=72 / 0=1,075)	1.14 (0.93-1.41)	0.201	1.09 (0.89-1.35)	0.398
Meta-analysis	(1=373 / 0=7,754)	1.27 (1.18-1.36)	$5.09*10^{-10}$	1.21 (1.11-1.30)	$2.87*10^{-6}$

* Adjusted for age, sex, HDL cholesterol, triglycerides, BMI, hypertension and glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL = reference)

† Adjusted for age, sex, HDL cholesterol, triglycerides, BMI, hypertension and logarithmized glucose concentrations

Supplementary Table 8: 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) and family history of diabetes.

Individuals with incident type 2 diabetes (n=107)				
		Baseline model plus afamin		
Baseline model	Total	<5% risk	5-24% risk	>=25% risk
<5% risk	14	10 (71.4)	4 (28.6) *	0 (0.0) *
5-24% risk	62	2 (3.2) †	46 (74.1)	14 (22.6) *
>=25% risk	31	0 (0.0) †	3 (9.7) †	28 (90.3)
Total	107	12	53	42

* Moved to higher risk which is correctly reclassified (light gray), n =18; † Moved to lower risk which is wrongly reclassified (dark gray), n =5; stayed in the same risk category (medium grey), n=84; **NRI 0.121 (95%CI 0.037-0.206), p=0.005.**

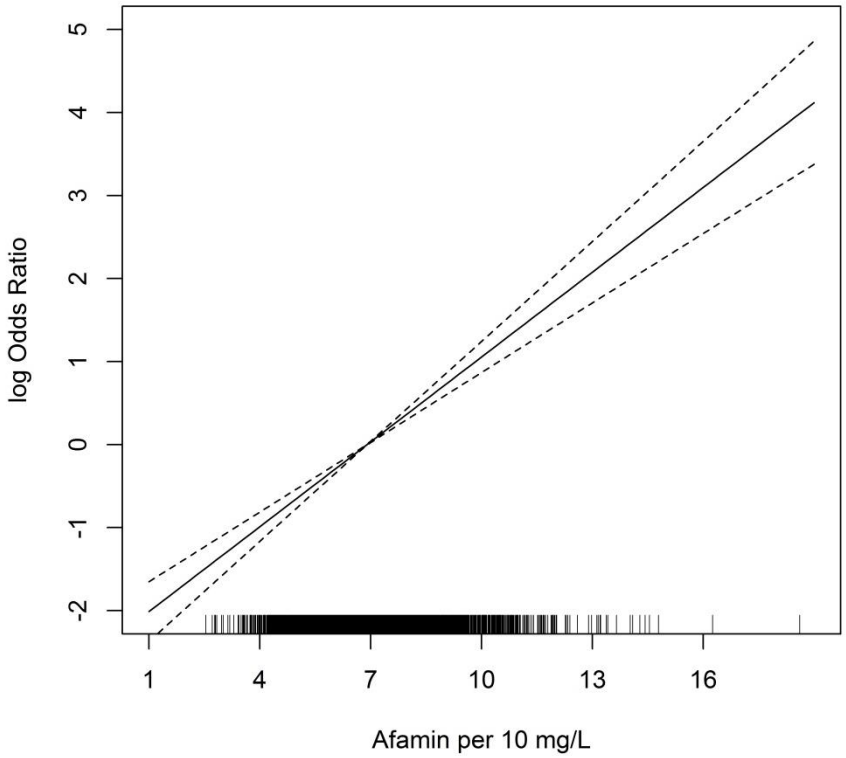
Individuals without incident type 2 diabetes (n=1,563)				
		Baseline model plus afamin		
Baseline model	Total	<5% risk	5-24% risk	>=25% risk
<5% risk	1,137	1,097 (96.5)	39 (3.4) †	1 (0.09) †
5-24% risk	355	61 (17.2) *	272 (76.6)	22 (6.2) †
>=25% risk	71	0 (0.0) *	20 (28.2) *	51 (71.8)
Total	1,563	1,158	331	74

* Moved to lower risk category which is correctly reclassified (light gray), n =81; † Moved to higher risk category which is wrongly reclassified (dark gray), n=62; stayed in the same risk category (medium grey); n = 1,420; **NRI 0.012 (95%CI -0.003-0.027), p=0.115.**

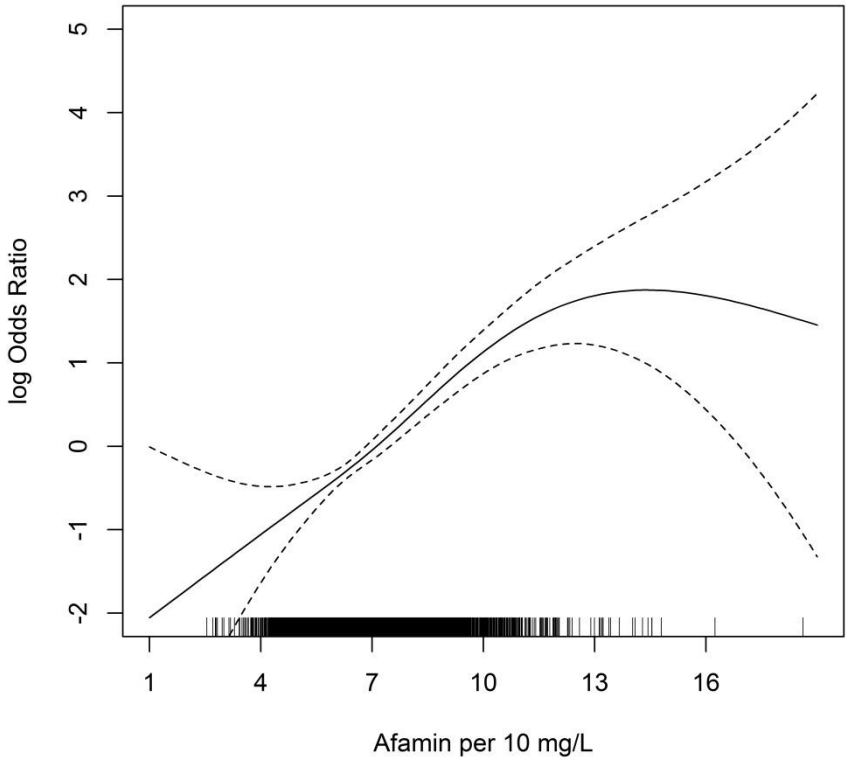
Values are presented as n (row percent).

Categorical net reclassification improvement (NRI) in this table is calculated for 107 individuals with and for 1,563 individuals without type 2 diabetes. **Overall NRI for the total group: 0.134 (95%CI 0.044-0.223), p=0.003.**

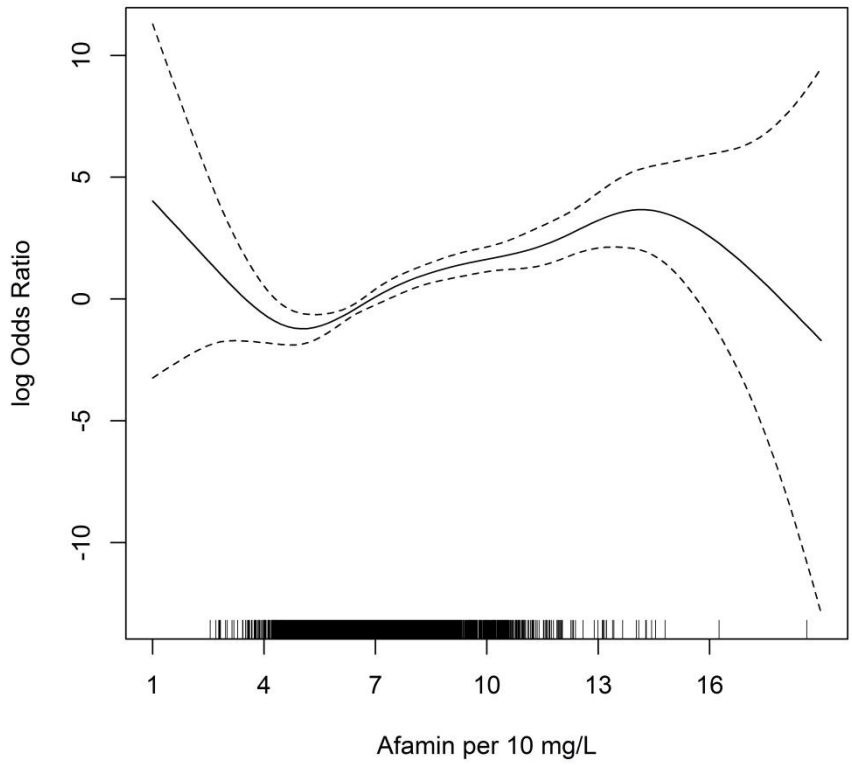
Supplementary Figure 1: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on prediabetes in the age and sex-adjusted logistic regression model in KORA F4. The dashed lines correspond to 95% confidence bands.



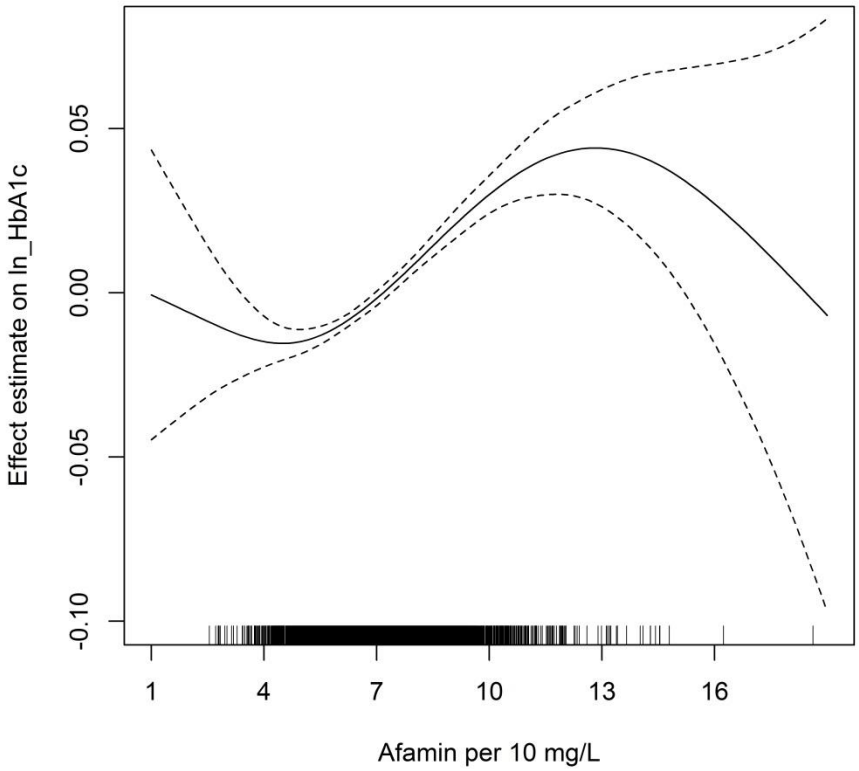
Supplementary Figure 2: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on prevalent type 2 diabetes in the age- and sex-adjusted logistic regression model in KORA F4. The dashed lines correspond to 95% confidence bands.



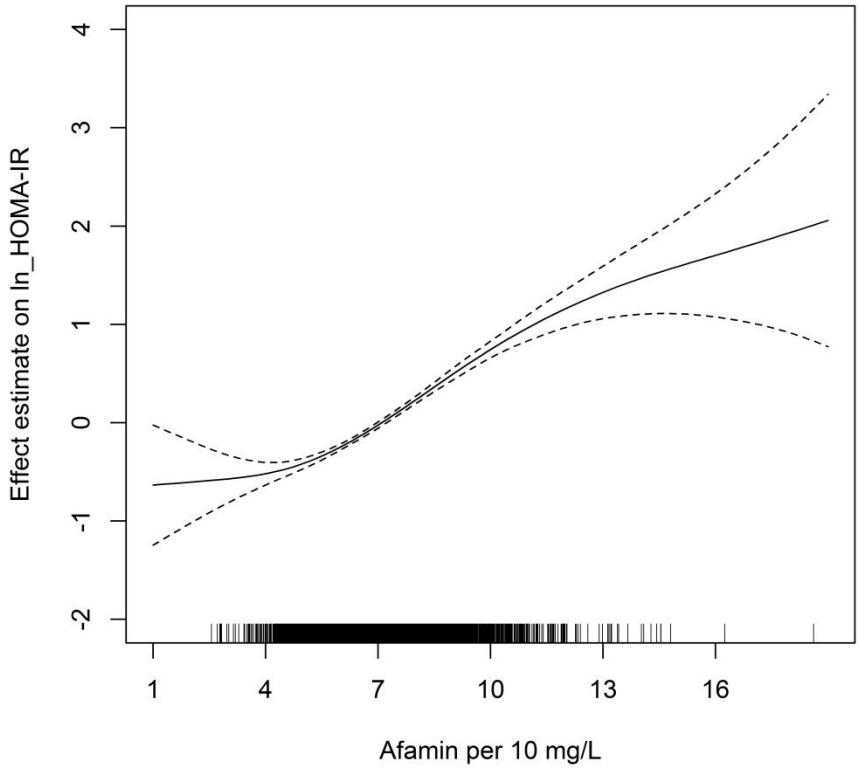
Supplementary Figure 3: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on incident type 2 diabetes in the age- and sex-adjusted logistic regression model in KORA F4. The dashed lines correspond to 95% confidence bands.



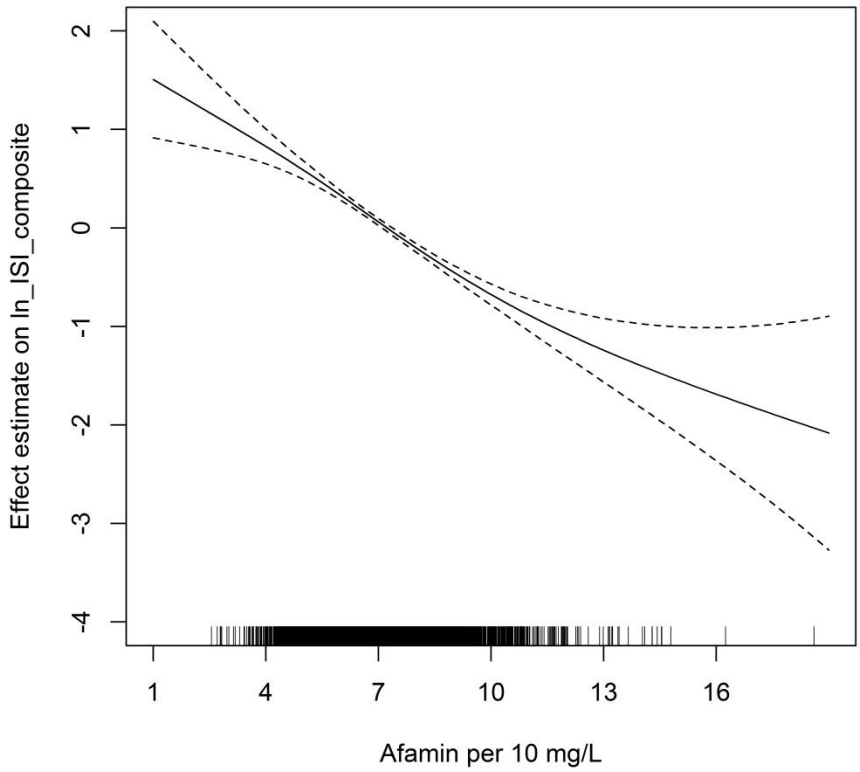
Supplementary Figure 4: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on logarithmized HbA1c in the age- and sex-adjusted linear regression model in KORA F4 in those without type 2 diabetes at baseline. The dashed lines correspond to 95% confidence bands.



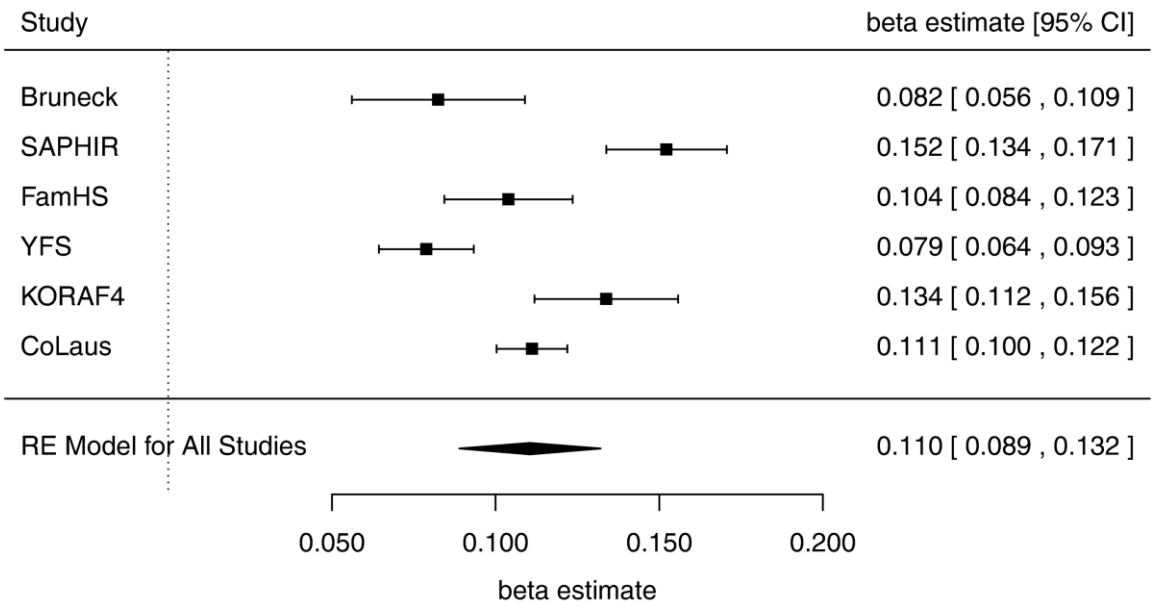
Supplementary Figure 5: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on logarithmized HOMA-IR in the age- and sex-adjusted linear regression model in KORA F4 in those without type 2 diabetes at baseline. The dashed lines correspond to 95% confidence bands



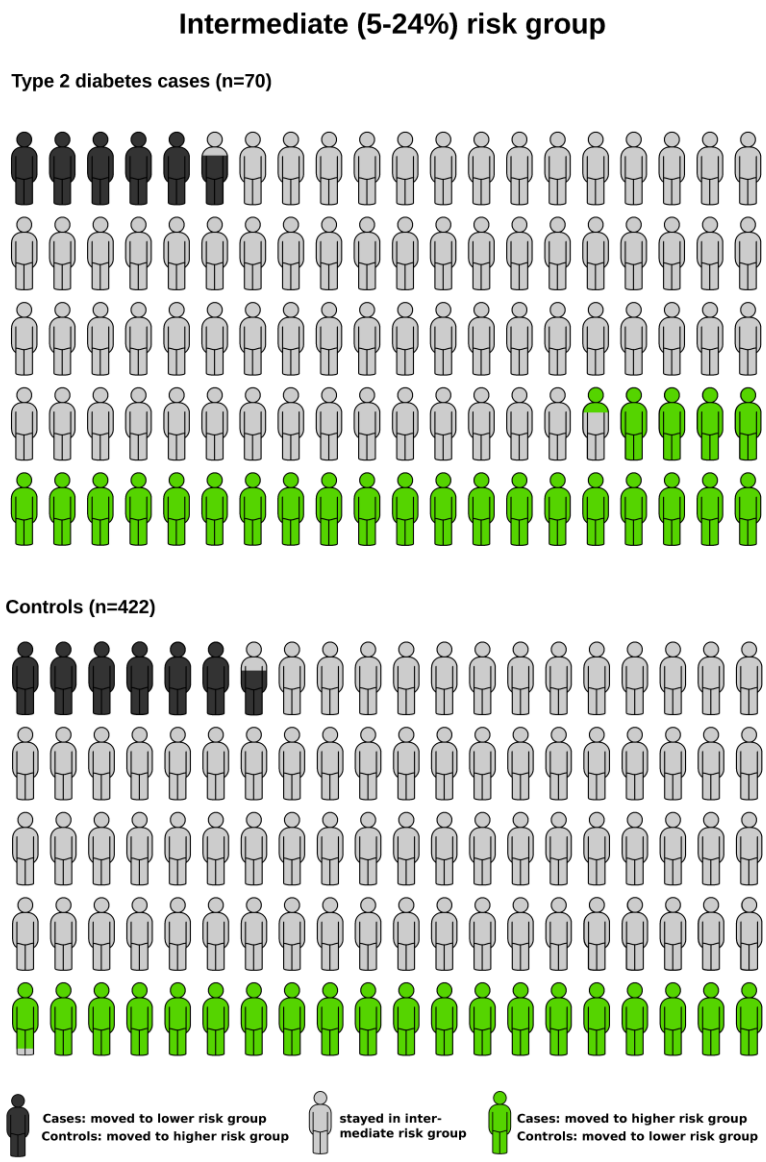
Supplementary Figure 6: Nonlinear P-Spline for afamin concentrations (per 10 mg/L) on logarithmized whole-body ISI(composite) in the age- and sex-adjusted linear regression model in KORA F4 in those without type 2 diabetes at baseline. The dashed lines correspond to 95% confidence bands



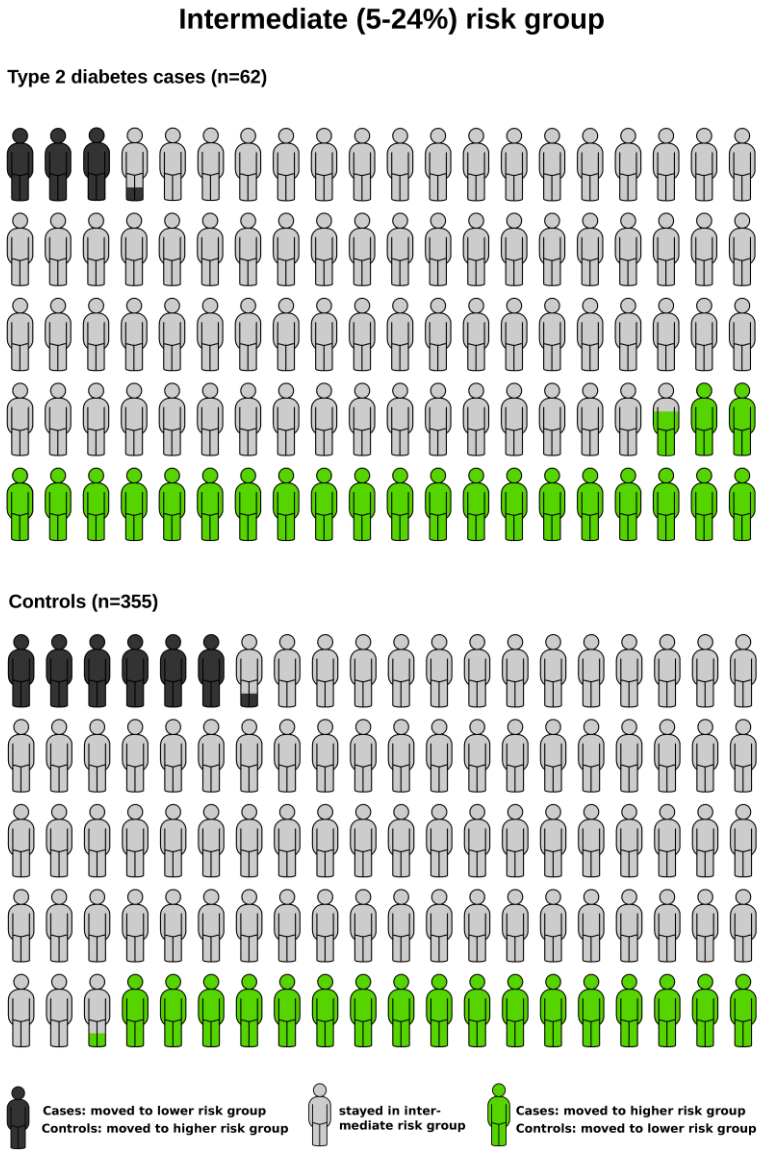
Supplementary Figure 7: Forest plot illustrating the association of afamin (increment 10 mg/L) with logarithmized insulin resistance index (HOMA-IR) (extended adjustment model), based on a random effects (RE) model for all 6 studies with available HOMA-IR measurements. Beta estimates and 95% confidence intervals are shown for each study and the pooled analysis.



Supplementary Figure 8: Reclassification of individuals (70 cases with type 2 diabetes and 422 controls) predicted to be at intermediate risk (5-24%) for the development of type 2 diabetes during follow-up (median 6.4 years) based on an additional inclusion of afamin concentrations in the KORA F4 extended risk model as compared to a risk model including age, sex and major metabolic risk factors or parameters (HDL cholesterol, triglycerides, BMI, hypertension, and fasting plasma glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference)). Adding afamin to the risk model resulted in a reclassification of 30.0% of patients and 26.5% of controls. Proportions are shown for 1) type 2 diabetes cases (70.0%) and controls (73.5%) that stayed in the intermediate risk group (illustrated in grey), and 2) type 2 diabetes cases that were correctly reclassified and thus moved to a higher risk category (24.3%) and controls that moved to a lower risk category (19.9%), respectively (illustrated in green) and 3) type 2 diabetes cases that were wrongly reclassified and thus moved to a lower risk category (5.7%) and controls that moved to a higher risk category (6.6%), respectively (illustrated in black).



Supplementary Figure 9: Reclassification of individuals (62 cases with type 2 diabetes and 355 controls) predicted to be at intermediate risk (5-24%) for the development of type 2 diabetes during follow-up (median 6.4 years) based on an additional inclusion of afamin concentrations in the KORA F4 extended risk model as compared to a risk model including age, sex and major metabolic risk factors or parameters (HDL cholesterol, triglycerides, BMI, hypertension, plasma glucose concentrations ≥100 mg/dL (100-125 mg/dL vs. <100 mg/dL=reference) and family history of diabetes). Adding afamin to the risk model resulted in a reclassification of 25.8% of patients and 23.4% of controls. Proportions are shown for 1) type 2 diabetes cases (74.1%) and controls (76.6%) that stayed in the intermediate risk group (illustrated in grey), and 2) type 2 diabetes cases that were correctly reclassified and thus moved to a higher risk group (22.6%) and controls that moved to a lower risk group (17.2%), respectively (illustrated in green) and 3) type 2 diabetes cases that were wrongly reclassified and thus moved to a lower risk group (3.2%) and controls that moved to a higher risk group (6.2%), respectively (illustrated in black).



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