Insulin-like growth factor binding protein 2 (IGFBP-2) and the risk of developing type 2 diabetes

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Abstract

Recent studies suggest that insulin-like growth factor binding protein-2 (IGFBP-2) may protect against type 2 diabetes but population-based human studies are scarce. We aimed to investigate the prospective association of circulating IGFBP-2 concentrations and of differential methylation in the *IGFBP-2* gene with type 2 diabetes risk.

Within the EPIC-Potsdam cohort (n=27,548), circulating IGFBP-2 concentration was assessed in a nested case-cohort (random subcohort, n=2500, all incident type 2 diabetes cases, n=820). A nested 1:1 matched case-control sample (300 incident type 2 diabetes cases, 300 controls) was constructed for DNA-methylation profiling. Longitudinal associations were evaluated in Cox models (case-cohort) and conditional logistic models (case-control), adjusting for age, sex, anthropometry, lifestyle and a large set of type 2 diabetes-related biomarkers.

Higher circulating IGFBP-2 concentrations (median 92 ng/mL) were cross-sectional linked to lower BMI, waist circumference, fatty liver index, triglycerides, fetuin A, ALT and γ-GT, and longitudinal associated with lower type 2 diabetes risk (HR per SD 0.65, 95%CI 0.53, 0.8). A methylation score based on seven type 2 diabetes-related CpGs in the *IGFBP-2* gene was associated with higher type 2 diabetes risk (OR per SD 2.7, 95%CI 2.1, 3.5).

Our results are consistent with a type 2 diabetes-protective effect of high circulating IGFBP-2 concentration, and suggest that epigenetic silencing of the *IGFBP-2* gene might predispose for type 2 diabetes.

The insulin-like growth factor (IGF)-axis regulates proliferation and differentiation processes, and modulates metabolic pathways. For example, IGF-1, the major circulating IGF, stimulates peripheral glucose uptake, lipogenesis and glycogen synthesis (1-3). Observation studies in human populations linked the IGF-axis to the metabolic syndrome and type 2 diabetes (4-8). The fifteen so far identified IGF-binding proteins (IGFBPs) importantly impact on systemic IGF-signaling by modulating activity and decay of their binding partners. Moreover, some IGFBPs exhibit IGF-independent signaling functions.

Recent evidence suggests beneficial effects of IGFBP-2 on systemic metabolism. IGFBP-2 may inhibit adipogenesis and enhance long-term insulin sensitivity (9), partly through interaction with IGF-1 signaling. Moreover, IGFBP-2, which is mainly released by the liver (10), directly supports glucose homeostasis, e.g. by stimulating glucose uptake into adipocytes in an IGF-independent manner (11, 12). Accordingly, IGFBP-2 overexpression was demonstrated to ameliorate insulin resistance in obese mice (13). To date, the only population-based study on IGFBP-2 and incident type 2 diabetes was conducted in a large prospective cohort of women. Higher circulating IGFBP-2 concentrations were strongly associated with lower type 2 diabetes risk (7). We are not aware of prospective cohort studies on the relation between IGFBP-2 and type 2 diabetes risk in men.

Besides direct effects of circulating levels, IGFBP-2 may also play a role in the developmental origins of type 2 diabetes. For example, IGFBP-2 was implicated in childhood obesity (14). Moreover, human IGFBP-2-overexpressing transgenic mice were protected from diet-induced obesity and insulin resistance (12), while epigenetic variation links IGFBP-2 to liver fat accumulation in mice and in humans (15, 16). Taken together, relative IGFBP-2 deficiency may favor visceral adiposity and ectopic lipid storage particularly in the liver, which are established risk factors for type 2 diabetes.

Epigenetic alterations including DNA methylation emerge as an important determinant of the metabolic syndrome (17, 18). Interestingly, human (19-22) and animal studies (23, 24) indicate that several genes involved in the IGF-1 axis are highly regulated by epigenetic factors. Only two studies reported alterations in DNA methylation of the *IGFBP-2* gene. Methylation of single cytosine-phosphate-guanin sites (CpG) located in the intronic region of *Igfbp-2* gene in the liver was linked to development of obesity and elevated hepatic fat storage in mice (15). Similarly, in humans, hepatic hypermethylation in the homologous CpG site was associated with non-alcoholic fatty liver disease (NAFLD) (16). Thus, epigenetic repression of the *IGFBP-2* gene may facilitate body weight gain and hepatic lipid accumulation, thereby predisposing for type 2 diabetes development. A targeted investigation of DNA methylation of the *IGFBP-2* gene in relation to type 2 diabetes risk was not yet conducted in humans.

We hypothesize that [1] reduced IGFBP-2 plasma levels predict later development of type 2 diabetes and that [2] epigenetic silencing by differential methylation of the *IGFBP-2* gene might predispose for type 2 diabetes incidence. We measured circulating concentrations of the IGFBP-2 protein in a large prospective human population study. In addition, *IGFBP-2* DNA methylation in whole blood cells was analyzed as a surrogate measure of epigenetic regulation. We evaluated the link between IGFBP-2 concentration in the circulation and *IGFBP-2* DNA methylation levels with type 2 diabetes incidence.

Methods

Study population

The present study was conducted in the EPIC Potsdam cohort, comprising 27,548 participants (16,644 women and 10,904 men). Participants within an age-range of 35-65 years were recruited from the general population between 1994 and 1998 (25). The vast majority (>99%) of EPIC-Potsdam participants are of central European ancestry. At baseline, anthropometric

measures and blood samples were taken by qualified medical personnel, and lifestyle and dietary habits, sociodemographic characteristics, and current health status were assessed with validated, interviewer-assisted questionnaires. In terms of active follow-up, participants were contacted every 2-3 years. Response rates ranged between 90% and 96% per follow-up round (26). This study included follow-up information until 31st of August 2005 (censoring date). All participants gave informed consent to use their data for biomedical research and the study was approved by the Ethics Committee of the State of Brandenburg, Germany.

For efficient molecular phenotyping, a nested case-cohort was constructed, consisting of a random sample of all participants who provided blood (subcohort, n=2,500), and all incident type 2 diabetes cases that occurred until the censoring date (n=820). In line with the case-cohort design, there was an overlap of 94 cases that were also part of the subcohort. For the current analyses we excluded participants with prevalent or unclear type 2 diabetes-status at baseline or missing follow-up information or without sufficient blood samples (n=180), and with missing values for lifestyle- and biomarker-covariables (n=268). Thus the analytical sample comprised 2,778 participants, including 2,108 members of the subcohort and 755 type 2 diabetes cases with an overlap of 85 participants.

For DNA methylation profiling, a nested case-control study was constructed based on the case-cohort described above. From this source, 300 incident type 2 diabetes cases were randomly selected (27). The following matching criteria were applied: age (±6 months), sex, and fasting time (<3h, 3h to <6h, and ≥6 h before blood draw), time of day of blood sampling (±2 h), and season at blood sampling. Based on these criteria, each case was individually matched to 1 nondiabetic control, which was drawn from participants that had at least the diabetes-free follow-up time of the respective case (incidence density sampling). Ten pairs were excluded from analyses because DNA-samples did not pass quality control for methylation profiling.

Detection of incident type 2 diabetes cases

Systematic information sources for incident cases were self-report of a type 2 diabetes diagnosis, type 2 diabetes-relevant medication, and dietary treatment due to type 2 diabetes during follow-up. Furthermore, additional information was obtained from death certificates or from random sources, such as tumor centers, physicians, or clinics that provided assessments from other diagnoses. Once a participant was identified as a potential case, disease status was further verified by sending a standard inquiry form to the treating physician. Only physician-verified cases with a diagnosis of type 2 diabetes (International Classification of Diseases, 10th revision code: E11) and a diagnosis date after the baseline examination were considered confirmed incident cases of type 2 diabetes.

Quantification of circulating IGFBP-2

Baseline blood samples were collected in monovettes containing 10% citrate. Samples were fractioned and plasma was stored in tanks of liquid nitrogen (approximately -196°C) or deep freezers (-80°C) (28). Commercial sandwich ELISAs were used to quantify plasma concentrations of IGFBP-2 (RD systems, DY674), IGF-1 and IGFBP-3 (BioVendor Laboratorní medicína a.s., Brno, Czech Republic), and of adiponectin (LINCO Research, St. Charles, Missouri, USA); HDL-cholesterol, triglycerides, glucose, HbA1c and CRP were measured using an automatic ADIVA® 1650 analyzer (Siemens Medical Solutions, Erlangen, Germany). Details regarding the biomarker measurements were described elsewhere (8, 29).

DNA methylation analysis

For assessment of DNA methylation in whole blood cells, genomic DNA was extracted from buffy coat using Kit II. An amount of 750 ng of genomic DNA from each participant was bisulfite-converted using Zymo EZ-96 DNA Methylation™ (Zymo Research Corporation,Irvine,CA,USA) and then hybridized on Infinium® MethylationEPIC BeadChip, [Illumina (SanDiego)]. The Illumina, EPIC chip covered 890,703 cytosine positions located in

TSS200, TSS1500, 5UTR, 1Exon, gene body, 3UTR and intergenic regions of the human genome. For our analysis, all CpG sites covered by the MethylationEPIC BeadChip (33 CpGs) were considered, which were located in the IGFBP-2 gene region (chr2: 217496919-217528830).

Preprocessing and normalization of the raw methylation data included steps of probe filtering, color bias correction, background subtraction and beta-mixture quantile normalization and was processed with the R-package "ChAMP" as previously described (30, 31). To exclude batch effects the "champ.runCombat" function was consecutively used and reviewed with singular value decomposition method using the "champ.SVD" function. Thus, components selected in our analysis were independent from all covariates related to technical errors. Next, methylation data was corrected for cell type heterogeneity between samples by using "champ.refbase" (32). Probes annotated to contain SNPs were excluded. DNA methylation data of all CpG sites annotated to *IGFBP-2* gene were considered for analysis. Data from the ENCODE-Project (33) were used to identify hepatic H3K27ac, H3K4me3, H3K9ac histone marks in the *IGFBP-2* gene (*Supplementary Note 1*).

Statistical methods

The longitudinal association of IGFBP-2 with time-to-diabetes incidence was evaluated in Cox proportional hazards regression models according to the Prentice method for case-cohorts with age as underlying time scale. Study entry was defined by age at recruitment. Study exit was determined by age at diagnosis of diabetes, drop out or censoring, whichever came first. Adjustment variables were selected based on prior known relevance for type 2 diabetes risk. A minimal model was adjusted for age (strata variable) and sex. A second model was further adjusted for waist circumference, prevalence of hypertension, education [vocational training or lower, technical college, university] and lifestyle variables (leisure-time physical activity [sum of sports, biking, and gardening in h/week], smoking status [never smoker, ex-smoker

<20 units/day, ex-smoker >= 20 units/day, smoker <20 units/day, smoker >=20 units/day], alcohol intake [six categories, 1: <=6 g/day, 2: >6-12 g/day, 3: >12-24 g/day, 4: >24-60 g/day, 5: woman >60 g/day, men >60-96 g/d, 6: >96 g/day]). In a third model other components of the IGF-axis (IGF-1 and IGFBP-3) were additionally included. The fourth model further included the Fatty Liver Index (FLI) according to Bedognie et al., which was calculated based on waist circumference, BMI, and blood concentration of triglycerides and γ-Glutamyltransferase (γ-GT) as described elsewhere (34). The fifth model was additionally adjusted for established type 2 diabetes-related biomarkers (adiponectin, fetuin A, triglycerides, ALT, CRP, γ-GT). Glucose and HbA1c were further included in the sixth model.

The longitudinal association of *IGFBP-2* DNA methylation with type 2 diabetes incidence was investigated in conditional logistic regression models. Models were adjusted for alcohol intake, smoking status, and leisure-time physical activity. Age and sex were considered by design (matching variables). A methylation score was built based on the differentially methylated CpGs that were significantly associated with type 2 diabetes risk after correcting for multiple testing. For construction of the score, betas from a logistic regression with all included CpGs as exposure and type 2 diabetes as outcome were used as weights. The association of the score with type 2 diabetes risk was investigated using conditional logistic regression.

Statistical analyses were performed with SAS (version 9.4) and R (version 3.3.2). A type I error probability (p-value) <.05 was considered statistically significant. Where applicable, multiplicity of tests was considered by controlling the false discovery rate according to Benjamini and Hochberg (35).

Results

Baseline characteristics & correlation structure

Baseline characteristics of the study population are shown in **Table 1**. The median IGFBP-2 concentration was 92 ng/mL (IQR: 59-129 ng/mL). Five subgroups (Q1-Q5) were defined based on quintiles of the distribution of IGFBP-2 concentrations in the subcohort. Median BMI, waist circumference, FLI, triglycerides, fetuin A, ALT and γ-GT were lower in subgroups with higher IGFBP-2 concentrations. Median age was higher in subgroups with higher IGFBP-2 concentrations. **Figure 1** visualizes the semipartial correlation between plasma IGFBP-2 and established type 2 diabetes-related circulating biomarkers. Controlling for age, sex, and waist circumference, IGFBP-2 was weakly correlated ($0.1 < |r| < 0.3$) with higher adiponectin concentrations and with lower FLI and lower concentrations of triglycerides, fetuin A, ALT, γ-GT and CRP (**Fig. 1**).

IGFBP-2-related type 2 diabetes risk

Type 2 diabetes risk according to plasma IGFBP-2 concentrations is shown in **Table 2**. Controlling for age and sex, risk of diabetes incidence was substantially lower in participants with higher IGFBP-2 concentrations (HR per SD 0.28, 95%CI 0.24, 0.34). Adjusting for anthropometric parameters and lifestyle factors (Model 2), IGF-1 and IGFBP-3 (Model 3), FLI (Model 4), diabetes-related biomarkers (Model 5) and markers of glucose homeostasis (Model 6) in a stepwise manner attenuated the inverse association of IGFBP-2 with type 2 diabetes risk. Still, in the comprehensively adjusted Model 6 higher IGFBP-2 concentrations were associated with considerable and statistically significant lower diabetes risk (HR 0.65, 95%CI 0.53, 0.8). In stratified analyses, lower diabetes risk related to higher IGFBP-2 concentration was observed in men and in women, with a more pronounced inverse association in men (**Table 2**).

In restricted cubic spline analyses, a non-linear model significantly improved the model fit (*Supplementary Fig. 1*). The inverse association of IGFBP-2 with type 2 diabetes risk was steeper in participants below the 60th percentile (105 ng/mL) of the IGFBP-2 distribution and tended to level out in participants with high plasma IGFBP-2 concentrations. This functional form was consistent between men and women (*Supplementary Fig. 1*). Still, the general trend of lower type 2 diabetes risk with higher IGFBP-2 was consistent over the full observed range of concentrations, which justifies reporting of the linear effect estimates.

Regression diagnostics did not indicate violation of the proportional hazards assumption (*Supplementary Figs. 2 & 3*). Neither excluding participants for whom type 2 diabetes was diagnosed within the first two years of follow-up; nor excluding participants with high HbA1c (≥5.7%); nor restricting the analysis to non-fasted participants substantially changed the results (*data not shown*).

DNA methylation in the *IGFBP-2* and type 2 diabetes risk

Taking advantage of the high resolution of Illumina Human Methylation BeadChips, DNA methylation levels of thirty-three CpG sites annotated to the *IGFBP-2* gene were analyzed. We evaluated the association of DNA methylation at these CpGs with type 2 diabetes risk. In a first step, the single CpGs were considered as exposure and linked to diabetes incidence in logistic models. After correcting for multiple testing, seven CpGs were statistically significantly associated with type 2 diabetes risk (**Table 3,** *Supplementary Table 1*). As expected all CpGs located in CpG-island shore exhibited low degree of DNA methylation (eg. cg03625261, cg26187237, cg25316969) and those located 25 kb downstream the CpG-island showed intermediate to high levels of methylation (**Fig. 2**).

Hypermethylated CpGs in cases were exclusively located in the promoter region in proximity to active histone marks, whereas two of three CpGs that were hypomethylated in cases were located in or close to the gene body (**Fig. 2**, **Table 3**). The link between epigenetic variation

196 in the *IGFBP-2* gene and type 2 diabetes risk was summarized in a weighted *IGFBP-2* DNA
197 methylation score. Higher methylation score points were associated with a substantially
198 higher risk of diabetes incidence (OR per SD higher methylation score 2.7, 95%CI 2.1, 3.5)
199 (**Table 4**). In cross-sectional analyses stratified by case status, the methylation score points
200 were not associated with circulating IGFBP2 concentrations (log-transformed). The
201 standardized betas (p-values) were 0.04 (0.1) and -0.01 (0.8) in cases and controls,
202 respectively, in a model adjusted for age, sex, and lifestyle variables. Adjustment for
203 circulating IGFBP-2 concentration, waist circumference, FLI, and HbA1c and random
204 glucose, respectively, did not attenuate the relation between methylation score and type 2
205 diabetes risk (**Table 4**). Again, these results were highly robust in sub-analyses restricted to
206 participant with at least 2 years type 2 diabetes-free survival time and HbA1c levels <5.7%
207 (data not shown).

208 **Discussion**

209 We found strong inverse associations of circulating IGFBP-2 concentrations with type 2
210 diabetes risk in both sexes, which were robust against comprehensively controlling for
211 established phenotypic and metabolic risk factors. For example, type 2 diabetes risk was more
212 than doubled for participants with moderately low compared to participants with moderately
213 high circulating IGFBP-2 concentration (1 SD below vs. 1 SD above the mean). Moreover,
214 methylation levels of the *IGFBP-2* gene were also strongly linked to type 2 diabetes
215 incidence. After accounting for various other risk factors, the odds of developing diabetes was
216 more than 6 times higher for participants with a moderately high compared to those with a
217 moderately low methylation score (1 SD below vs. 1 SD above the mean). To our knowledge,
218 this is the first population-based prospective study on the relation between IGFBP-2 and type
219 2 diabetes risk in both sexes, and the first investigation of the longitudinal relation between
220 *IGFBP-2* DNA methylation and type 2 diabetes incidence.

Our finding of an inverse association of circulating IGFBP-2 with type 2 diabetes risk is consistent with the only previous prospective cohort study. Rajpathak et al. observed strong and robust associations of IGFBP-2 with type 2 diabetes risk within the Nurses' Health Study (NHS) (7). Women in the highest IGFBP-2 quintile-based group had a five-time lower type 2 diabetes risk compared those in the lowest group. In our study, the comprehensively adjusted effect estimate for four standard deviations higher IGFBP-2 concentrations also translates into an approximately five-time lower type 2 diabetes risk. Rajpathak et al. (7) raised the question whether their results in women were generalizable to men. Based on our findings in EPIC-Potsdam, this question can now be positively answered.

A possible explanation for the relation between circulating IGFBP-2 and type 2 diabetes risk is an involvement of IGFBP-2 in insulin-regulated pathways. A study in diabetes-prone mice demonstrated that adenovirus-mediated IGFBP-2 overexpression normalized insulin and glucose levels, and rescued mice from the metabolic consequences of impaired insulin signaling (13). These beneficial effects were also observed in animals with streptozocin-induced type 1 diabetes (13), which suggests that IGFBP-2 acts downstream of insulin. Moreover, improvements in glucose homeostasis were independent of changes in food intake and body weight (13). In vitro experiments showed that IGFBP-2 enhances GLUT4-mediated glucose uptake in adipocytes, suggesting direct interaction with insulin-signaling pathways (11). The whole body knockout of *IGFBP-2* had no impact on insulin sensitivity in young (eight and sixteen weeks old), non-challenged animals, which may be explained by compensatory upregulation of other IGFBPs (36). In summary, experimental model systems indicate that IGFBP-2 can have relevant influence on insulin-dependent pathways, but suggest that this regulatory potential of IGFBP-2 rather constitutes a compensatory system under challenged conditions.

Under non-experimental conditions, interpretation of the relation between circulating IGFBP-2 concentrations and type 2 diabetes risk is complicated because *IGFBP-2* expression is partly controlled by insulin (37) and cross-sectional associations of IGFBP-2 with insulin resistance were reported (38, 39). The aforementioned investigation in the NHS, however, found that the inverse association of IGFBP-2 with type 2 diabetes risk did not depend on fasting insulin concentrations, and was also detected in participants with both low baseline HbA1c and fasting insulin (7). Consistently, neither adjustment for glucose parameters, nor excluding participants with elevated baseline-HbA1c or with a type 2 diabetes diagnosis within the first two years of follow-up markedly attenuated the inverse association of IGFBP-2 with type 2 diabetes risk in EPIC-Potsdam. Thus, the link between IGFBP-2 and future type 2 diabetes incidence in human populations cannot be explained as consequence of already pathologically elevated insulin levels or undetected type 2 diabetes. The association structures are consistent with complex involvement of IGFBP-2 in type 2 diabetes pathogenesis, and suggest that high IGFBP-2 concentrations may protect against decompensation of systemic glucose metabolism.

Apart from the link of high circulating IGFBP-2 with low diabetes risk, we observed marked associations between *IGFBP-2* DNA methylation levels and type 2 diabetes risk. We found that a large proportion of the examined CpGs in the *IGFBP-2* gene (seven out of thirty-three) was associated with diabetes incidence. Hypermethylation is generally linked to gene silencing. This particularly applies for CpGs located in the promoter region as well as first exons and introns of the gene. Accordingly, type 2 diabetes risk was linked to hypermethylation of four CpGs located in the promoter, 5´UTR and exon1 region of the *IGFBP-2* gene. Interestingly, these 4 CpGs were in close proximity to enrichment of H3K27ac, H3K4me3 and H3K9ac. These active histone marks are known to be associated with high transcriptional states. In addition, our previous study indicated that a luciferase reporter construct of the homologous region in mice exhibits promoter activity (15). Taken

271 together, hypermethylation of CpGs located in such important cis regulatory region is likely
272 to interact with regulatory transcription factors and may affect *IGFBP-2* expression.

273 Hypomethylation of two CpGs sites (cg03149532; cg13220299) located in the end of intron 2
274 was linked to higher type 2 diabetes risk. Recent studies suggested that low levels of DNA
275 methylation within the gene body were related to inactive gene expression (40). The robust
276 association of the methylation score in sub-analyses excluding participants with an early
277 diabetes diagnosis and with elevated HbA1c levels make reverse causation through
278 undiagnosed type 2 diabetes unlikely. DNA methylation seems to be a stable marker. Thus,
279 the observed link of methylation of *IGFBP-2* with diabetes risk suggests that epigenetic
280 silencing of the *IGFBP-2* gene may predispose for onset of the disease. Still, our findings on
281 epigenetic alterations need to be interpreted with caution because we were the first to conduct
282 a targeted investigation of DNA methylation in the *IGFBP-2* gene region and incident type 2
283 diabetes.

284 DNA methylation in the *IGFBP-2* gene and circulating IGFBP-2 concentrations were not
285 associated in our adult study population. This was not unexpected based on our previous
286 experiments, where we showed that DNA methylation of *Igfbp-2* in the liver was different in
287 mice (C57BL/6) that were susceptible to diet-induced fatty liver (15). Hypermethylation of
288 *Igfbp-2* was related to lower plasma levels of IGFBP-2 at 6 weeks but not anymore at 20
289 weeks of age (15). Hence, the systemic consequences of *IGFBP-2* DNA methylation may
290 depend on the developmental phase. However, as we relied on a selected matched case-
291 control sample, we cannot necessarily generalize the cross-sectional null finding to the full
292 study population.

293 We further hypothesized that the effect of *IGFBP-2* DNA methylation on type 2 diabetes risk
294 might be mediated by early impairment of insulin signaling, and by facilitating visceral
295 adiposity and liver fat accumulation. However, conditioning the relation between methylation

296 score and type 2 diabetes risk on HbA1c and glucose only slightly attenuated the risk
297 estimate, and conditioning on waist circumference and FLI, respectively, resulted in stronger
298 risk estimates. These results do not support our a priori mediation hypotheses. We can still not
299 rule out the possibility that *IGFBP-2* DNA methylation may affect aspects of insulin
300 sensitivity and hepatic lipid metabolism that are less well captured by the phenotypic markers
301 that we used to reflect these physiological traits.

302 Importantly, the relations of *IGFBP-2* DNA methylation and of circulating IGFBP-2 with
303 type 2 diabetes risk were largely independent, i.e. the associations were only marginally
304 attenuated in mutually adjusted models. In our observational design, we cannot narrowly pin
305 down the actual biological dimension of IGFBP-2 signaling reflected by each of the
306 parameters that we used. The fact that both, IGFBP-2 concentrations in the circulation and
307 DNA methylation of *IGFBP-2* in blood cells, were independently related to type 2 diabetes
308 risk, however, likely implicates low IGFBP-2 availability in developmental and metabolic
309 processes that predispose for type 2 diabetes.

310 Our study had limitations. First, based on our observations we can only speculate on potential
311 causal paths which link IGFBP-2 to type 2 diabetes development. We used a prospective
312 cohort design, comprehensively adjusted for potential phenotypical and lifestyle confounders
313 and a large set of known type 2 diabetes-related biomarkers, and our results were robust in
314 sub-analyses restricted to participants who remained free of type 2 diabetes for at least 2 years
315 after recruitment and who did not have elevated HbA1c levels at baseline. Thus, our results
316 cannot be explained by confounding through known type 2 diabetes risk factors, and reverse
317 causation should not be an issue. Now, conclusive animal experiments and Mendelian
318 randomization studies may be applicable to elucidate the mechanisms that implicate IGFBP-2
319 in type 2 diabetes development and to clarify whether IGFBP-2 itself causally affects type 2
320 diabetes risk. Second, our findings regarding the association between DNA methylation in the

IGFBP-2 gene and type 2 diabetes risk warrant external validation. A strength of the incidence-density sampling is that the OR we provided should approximate relative risks in the full study population. Due to the matched case-control design, however, we cannot provide generalizable information on the cross-sectional association of methylation in the *IGFBP-2* gene with type 2 diabetes-related phenotypical traits and biomarkers. Third, in the population-based EPIC-Potsdam cohort we relied on indirect measures of liver fat accumulation and DNA methylation was measured in whole blood cells. Human studies that have access to other tissue samples (e.g. liver, adipose tissue) may reveal pathways that link *IGFBP-2* methylation to type 2 diabetes risk.

To conclude, we observed in a population-based human cohort study that circulating IGFBP-2 concentrations and DNA methylation levels within the *IGFBP-2* gene in blood cells were independently and both strongly associated with type 2 diabetes risk. The association structure in EPIC-Potsdam is consistent with a substantial role of impaired IGFBP-2 signaling in biological processes that predispose for type 2 diabetes incidence.

Article Information

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Author Contributions. The authors' responsibilities were as follows - CW, MO, MBS and AS: developed the project, designed the analysis plan, wrote the manuscript, and had primary responsibility for the final content; CW, OK, MO, PG and MJ conducted statistical analyses; AT, JK, HG, TP and HB contributed to the acquisition of data; CW, MO, KM, TP, MBS and AS contributed to the interpretation of data; and all authors contributed to revising the manuscript critically for important intellectual content and read and approved the final version of the manuscript. AS is guarantor of the study.

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Table 1: Baseline characteristics over IGFBP-2 quintile-based groups, EPIC-Potsdam Study (subcohort, n=2108)

	Subgroups according to quintiles of IGFBP-2 (Q1-Q5)					
	Q1* (n=422)	Q2 (n=421)	Q3 (n=422)	Q4 (n=421)	Q5 (n=422)	All
IGFB2 range [ng/mL]	41 (1-54)[†]	65 (54-78)	92 (78-105)	121 (105-142)	175 (142-666)	92 (59, 129)[‡]
Gender [female %]	67	63	62	60	60	62
Age at baseline [y]	45 (40, 54)	48 (41, 57)	51 (43, 59)	52 (43, 59)	54 (44, 60)	50 (42, 58)
BMI [kg/m²]	28 (25, 31)	27 (24, 30)	26 (23, 28)	25 (23, 27)	23 (21, 26)	26 (23, 28)
Waist [cm]	91 (79, 101)	88 (77, 96)	86 (77, 94)	83 (75, 91)	79 (71, 87)	85 (75, 94)
Activity [h/week]	4 (2, 7)	5 (2, 8)	5 (2, 8)	5 (2, 9)	5 (2, 9)	5 (2, 8)
Smoking Status [%]						
never smoker	45	49	51	50	46	48
ex-smoker, < 20 units/day	25	24	22	22	21	23
ex-smoker, ≥ 20 units/day	14	11	8	7	5	9
smoker, < 20 units/day	12	12	15	14	19	14
smoker, ≥ 20 units/day	3	5	4	7	9	6
Education [%]						
vocational training or lower	42	33	38	37	38	37
technical college	23	24	24	28	22	24
university	35	43	38	36	40	38
Alcohol [g/day]	8.5 (3.1, 20.6)	8.9 (3.4, 20.1)	7.3 (2.2, 16.9)	8.7 (3, 20.6)	6.9 (2.2, 16.5)	8.2 (2.6, 19.4)
IGF-1 [ng/mL]	162 (128, 206)	172 (137, 210)	167 (135, 203)	162 (135, 199)	150 (126, 184)	163 (133, 201)
IGFBP-3 [µg/mL]	3.1 (2.8, 3.5)	3.1 (2.8, 3.5)	3.1 (2.7, 3.5)	3 (2.7, 3.4)	3.1 (2.7, 3.5)	3.1 (2.7, 3.5)
Triglyceride [mg/dL]	133 (92, 199)	119 (83, 180)	106 (77, 158)	98 (69, 137)	88 (64, 129)	107 (77, 161)
Fetuin [µg/mL]	288 (248, 335)	266 (227, 312)	263 (220, 299)	258 (223, 301)	250 (212, 290)	265 (226, 307)
ALT [U/L]	22 (15, 37)	21 (15, 31)	20 (15, 28)	19 (14, 24)	17 (14, 22)	20 (15, 28)
γ-GT [U/L]	23 (13, 42)	19 (13, 40)	17 (12, 28)	14 (9, 26)	14 (9, 22)	17 (11, 31)
FLI	53 (16, 80)	39 (12, 67)	26 (10, 55)	19 (6, 41)	10 (5, 30)	26 (8, 58)
Adiponectin [µg/mL]	6.7 (4.9, 8.9)	7.2 (5.3, 9.8)	7.9 (5.8, 10.4)	8.6 (5.9, 11.7)	9.2 (6.5, 12.7)	7.8 (5.7, 10.7)
Glucose [mg/dL]	102 (94, 113)	101 (93, 110)	101 (96, 110)	102 (92, 110)	99 (91, 108)	101 (94, 110)
HbA1c [%]	5.5 (5.2, 5.8)	5.4 (5.1, 5.8)	5.4 (5.1, 5.7)	5.4 (5.1, 5.7)	5.4 (5.1, 5.7)	5.4 (5.1, 5.7)
HbA1c [mmol/mol]	36.1 (33, 40)	35.4 (32.2, 39.6)	35.4 (32.2, 39)	35.3 (32.3, 38.8)	35.3 (32.6, 38.5)	35.5 (32.5, 39.1)

*The representative subcohort was divided into five subgroups (Q1-Q5) according to quintiles of the distribution of IGFBP-2 plasma concentrations. [†]Median (minimum - maximum), all such values; [‡]Median (interquartile range), all such values.

Table 2: Association of plasma IGFBP-2 concentration with the risk of developing type 2 diabetes

	Pooled (n=2778, n_{cases}=755)		Men (n=1188, n_{cases}=444)		Women (n=1590, n_{cases}=311)	
	HR per SD*	(95%-CI)	HR per SD	(95%-CI)	HR per SD	(95%-CI)
Model 1	0.28	(0.24, 0.34)	0.3	(0.24, 0.38)	0.26	(0.19, 0.35)
Model 2	0.41	(0.34, 0.51)	0.41	(0.31, 0.53)	0.42	(0.31, 0.56)
Model 3	0.41	(0.33, 0.5)	0.39	(0.3, 0.52)	0.43	(0.32, 0.57)
Model 4	0.49	(0.4,0.6)	0.46	(0.35, 0.61)	0.52	(0.4, 0.69)
Model 5	0.56	(0.45, 0.68)	0.47	(0.35, 0.63)	0.7	(0.52, 0.93)
Model 6	0.65	(0.53, 0.8)	0.58	(0.43, 0.77)	0.81	(0.61, 1.07)

*Hazard ratio (HR) of developing type 2 diabetes associated with one standard (SD) higher circulating IGFBP-2 levels. Model 1: adjusted for age and if applicable for sex; Model 2: additionally adjusted for waist circumference, prevalence of hypertension, education, physical activity, smoking status, and alcohol intake; Model 3: additionally adjusted for IGF-1 and IGFPB-3; Model 4: additionally adjusted for FLI; Model 5: additionally adjusted for adiponectin, fetuin A, triglycerides, CRP, ALT, γ-GT, Model 6: additionally glucose and HbA1c.

Table 3: Association of DNA methylation of *IGFBP-2* with the odds of developing type 2 diabetes

Methylation of…	OR per SD[†]	(95%-CI)
CpGs* in the promoter region		
cg05689321	1.11	(1.05, 1.17)
cg26187237	1.57	(1.3, 1.9)
cg25316969	1.26	(1.1, 1.45)
cg03625261	1.36	(1.11, 1.66)
cg25380868	0.64	(0.53, 0.78)
CpGs in the gene body		
cg13220299	0.6	(0.45, 0.8)
cg03149532	0.86	(0.81, 0.92)

DNA methylation of the IGFBP-2 gene in relation to type 2 diabetes risk was evaluated in a 1:1 matched case-control study (analytical sample n=290 cases, and n=290 controls), nested within the EPIC-Potsdam cohort study; controls were drawn by incidence density sampling and matched for age (±6 months), sex, fasting time (<3h, 3h to <6h, and ≥6 h before blood draw), time of day of blood sampling (±2 h), and season at blood sampling.

**CpG*: DNA sequence where cytosine and guanine are connected by a single phosphate group (*5'—C—phosphate—G—3'*); CpG sites are subject to differential methylation; shown are all available CpGs in the *IGFBP-2* gene that were significantly differently methylated between cases and controls after correcting for multiple testing.

[†]Odds ratio (OR) of developing type 2 diabetes associated with one standard (SD) higher methylation adjusted for alcohol consumption, smoking, and physical activity as well as age and sex (matching variables).

Table 4: Methylation Score and type 2 diabetes risk

Exposure	Additionally adjusted for…	OR per SD*	(95%-CI)
Methylation score	-	2.71	(2.12, 3.46)
Methylation score	IGFBP-2	2.83	(2.1, 3.81)
Methylation score	Waist circumference	3.15	(2.25, 4.42)
Methylation score	Fatty Liver Index	3.25	(2.25, 4.68)
Methylation score	HbA1c and glucose	2.57	(1.83, 3.61)

DNA methylation of the IGFBP-2 gene in relation to type 2 diabetes risk was evaluated in a 1:1 matched case-control study (analytical sample n=290 cases, and n=290 controls), nested within the EPIC-Potsdam cohort study; controls were drawn by incidence density sampling and matched for age (±6 months), sex, fasting time (<3h, 3h to <6h, and ≥6 h before blood draw), time of day of blood sampling (±2 h), and season at blood sampling.

*Odds ratio (OR) of developing type 2 diabetes associated with one standard (SD) higher methylation score; all models were adjusted for alcohol consumption, smoking, and physical activity as well as age and sex (matching variables).

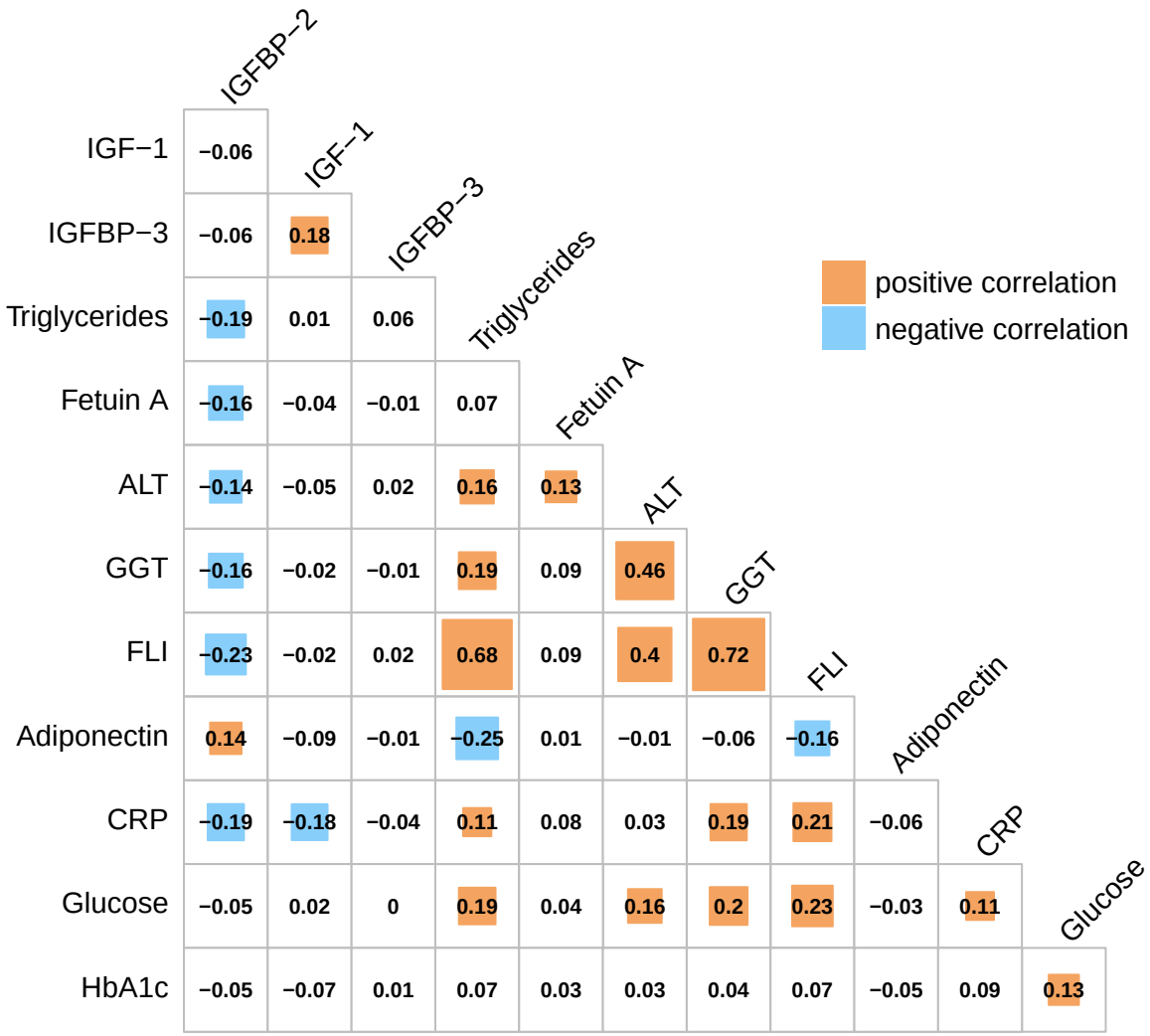
Figure 1

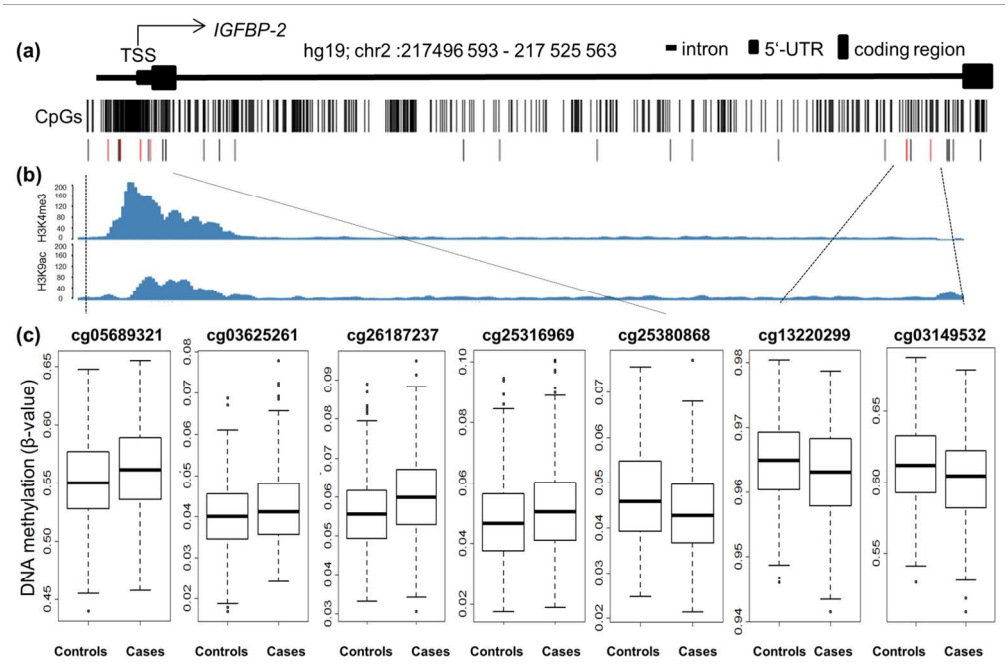
Correlation structure between IGFBP-2 and other type 2 diabetes-related biomarkers. Numbers indicate age-, sex-, and waist circumference-adjusted Spearman correlation coefficients. The size of the colored rectangles corresponds to the strength of correlations (complete color filling, absolute correlation coefficient $|r|=1$; no color filling, $|r|<0.1$).

Figure 2

Differential DNA methylation levels of *IGFBP-2* gene in cases and controls. Genomic organization of *IGFBP-2* gene 1500 bp upstream of the transcription start site (TSS) up to the end of exon 2 is shown in (a). All CpG sites located in this region (spanning from chr2: 217496 593 - 217 525 563) are indicated as lines in the upper part. Depicted in the lower part are CpGs covered by illumina array (black) and CpGs significantly different between cases and controls are highlighted in red. (b) Hepatic pattern of active histone marks H3K4me3 and H3K9ac (ENCODE data). (c) Boxplots of DNA methylation levels in cases and controls for CpGs that were statistically significantly differentially methylated between the two groups after controlling the false discovery rate.

Biomarker correlationmatrix (age−, gender− and waist−adjusted)





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127x85mm (300 x 300 DPI)

Online Supplementary Material

Insulin-like growth factor binding protein 2 (IGFBP-2) and the risk of developing type 2 diabetes

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Supplementary Note 1

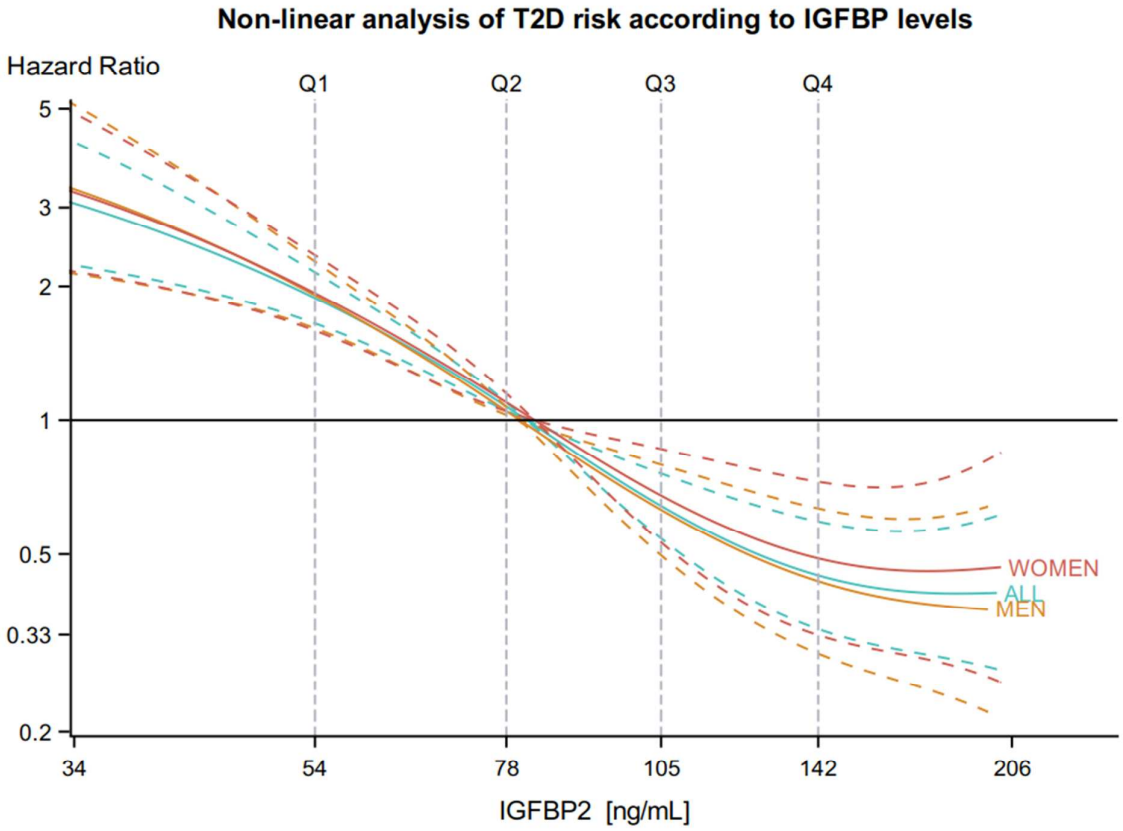
Analysis of cis regulatory elements of *IGFBP2* gene

For the identification of putative regulatory elements located within or in close proximity to the *IGFBP-2* gene, hepatic H3K27ac, H3K4me3, H3K9ac histone marks known to be associated with active promoters were downloaded from the ENCODE-Project. BAM-files were converted to bed files using BEDTools and then filtered for chromosomes. Bed-files were loaded into R (version 3.4.3) by use of Gviz (version 1.22.3), rtracklayer (version 1.38.3) and chipseq (version 1.28.0) packages. Chip-Seq scores were calculated within a size of 100 base pairs and plotted with the plot Tracks function. The resulting tracks of the single tissues were overlapped with help of Gimp (version 2.8).

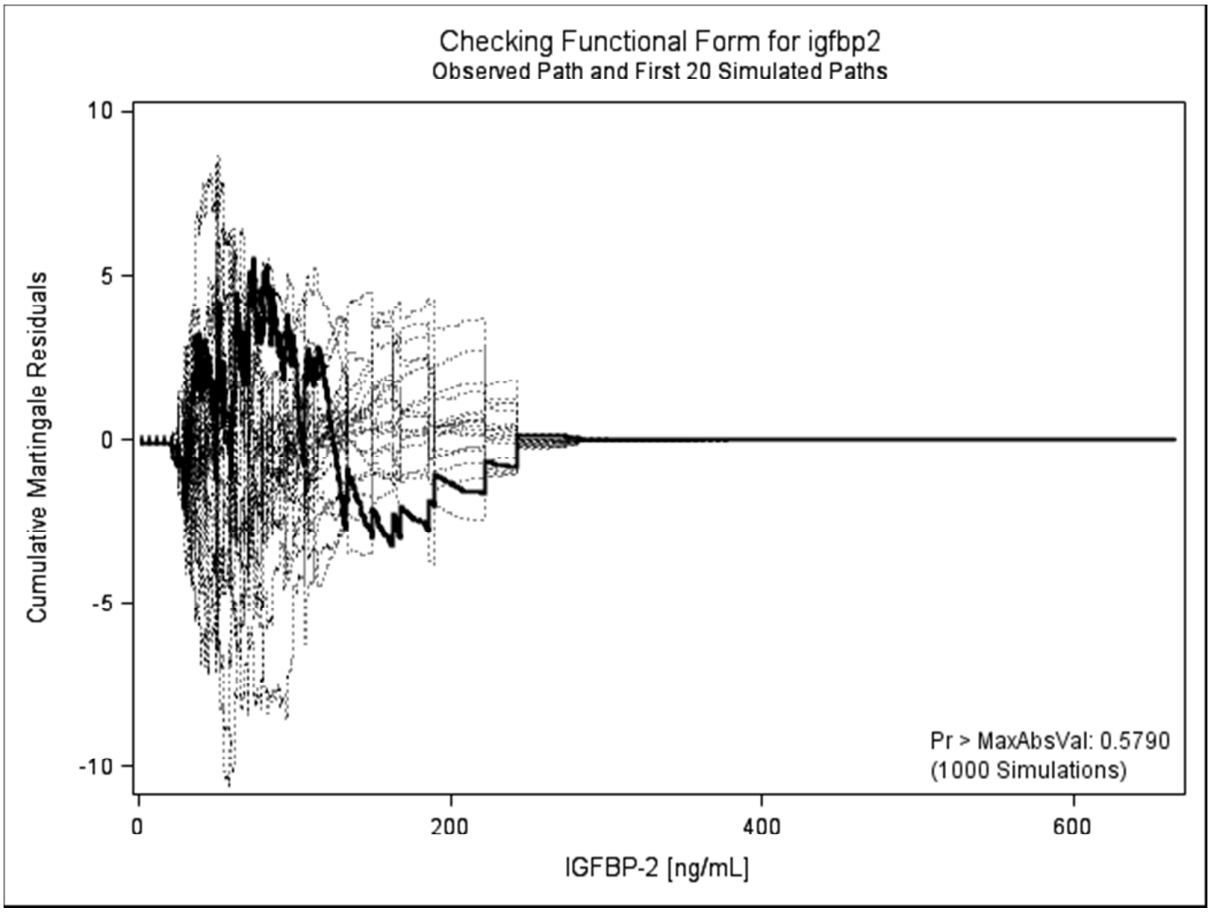
Supplemental Table 1: Baseline characteristics in cases and controls of the nested case-control sample with methylation data.

All (n=580)	**Controls** n=290	**Cases** n=290
IGFBP-2 [ng/ml]	100 (67, 142)	60 (44, 87)
Gender (% female)	48	48
Age at baseline [y]	56 (49, 60)	56 (49, 61)
BMI [kg/m²]	26 (24, 28)	30 (27, 33)
Waist [cm]	89 (81, 95)	100 (92, 107)
Smoking Status [%]		
never smoker	47	33
ex-smoker, < 20 u./d.	23	26
ex-smoker, ≥ 20 u./d.	11	19
smoker, < 20 u./d.	13	11
smoker, ≥ 20 u./d.	7	12
Education [%]		
vocational training or lower	37	47
technical college	24	22
university	39	31
Alcohol [g/day]	9.7 (3.3, 22.3)	8.2 (2.5 19.6)
FLI [%]	37 (13, 62)	79 (54, 90)
IGF-1 [ng/ml]	157 (126, 197)	153 (125, 194)
IGFBP-3 [µg/ml]	3 (2.7, 3.5)	3.1 (2.7, 3.5)
Triglyceride [mg/dl]	117 (84, 168)	168 (123, 241)
Fetuin [µg/ml]	257 (223, 302)	279 (236, 317)
GPT [U/L]	22 (16, 30)	29.5 (21, 42)
gamma-GT [U/L]	20 (13, 36)	34 (21, 53)
Adiponectin [µg/ml]	7.6 (5.4, 9.8)	5.3 (4, 7.5)
Glucose [mg/dl]	103 (95, 113)	118 (105, 140)
HbA1c [%]	5.4 (5.1, 5.7)	6.1 (5.7, 6.7)

Comparison of the baseline characteristics between randomly selected incident type 2 diabetes cases and 1:1 matched controls; matching criteria were age (±6 months), sex, and fasting time (<3h, 3h to <6h, and ≥6 h before blood draw), time of day of blood sampling (±2 h), and season at blood sampling.



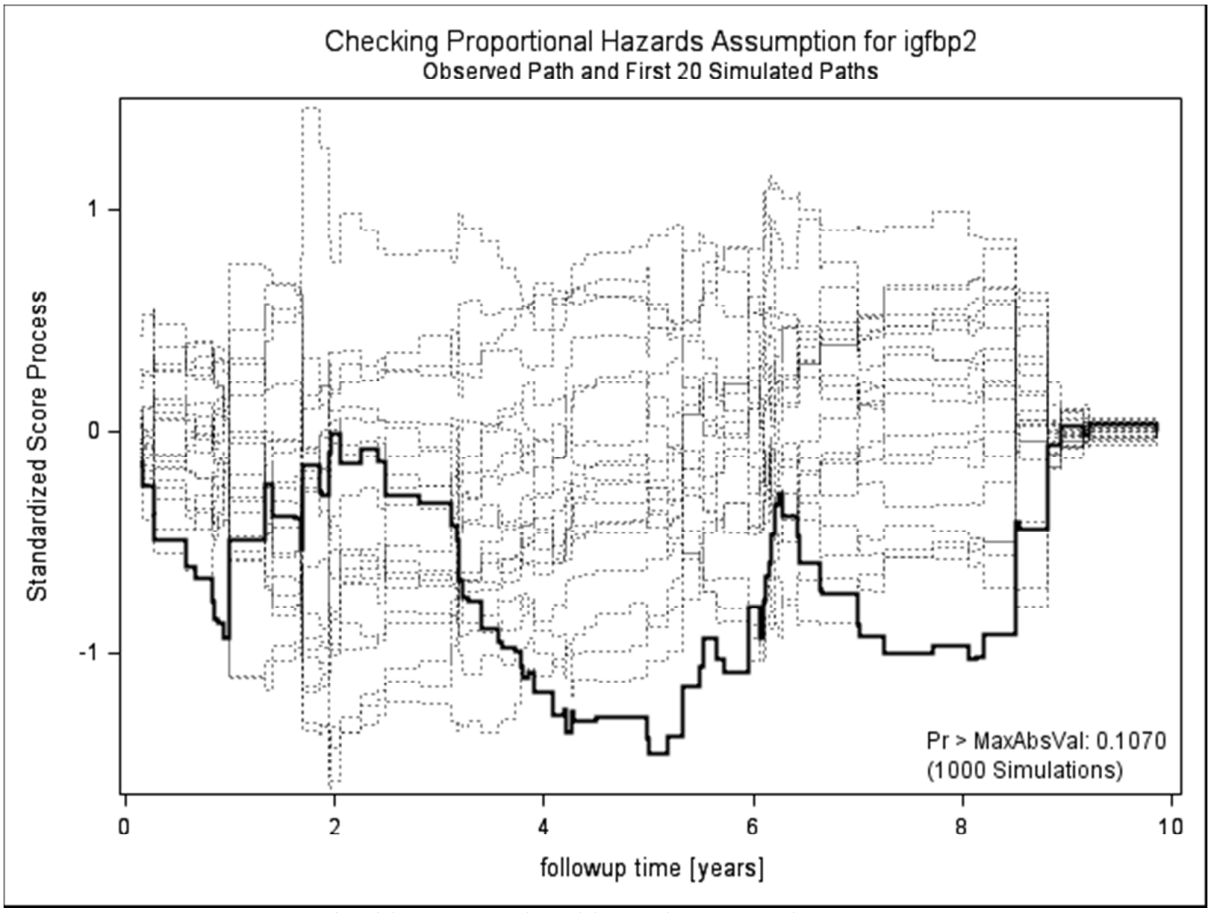
Supplemental Figure 1: Restricted cubic spline analysis of the non-linear relation between circulating IGFBP2 concentrations and type 2 diabetes (Q1-Q4: Quintiles of the IGFBP2 distribution)



Supremum Test for Functional Form

Variable	*Replications*	*Seed*	*Pr > MaxAbsVal*
igfbp2	1000	48680156	0.579

Supplemental Figure 2: Testing potential interactions with follow-up time



Supplemental Figure 3: Checking proportional hazards assumption