Insulin-Like Growth Factor Binding Protein 2 (IGFBP-2) and the Risk of Developing Type 2 Diabetes

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Diabetes 2019;68:188–197 | https://doi.org/10.2337/db18-0620

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 European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort (n = 27,548), circulating insulin-like growth factor binding protein 2 (IGFBP-2) concentration was assessed in a nested case-cohort (random subcohort, n = 2,500, all case subjects with incident type 2 diabetes, n = 820). A nested 1:1 matched case-control sample (300 case subjects with incident type 2 diabetes, 300 control subjects) 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-sectionally linked to lower BMI, waist circumference, fatty liver index (FLI), triglycerides, fetuin A, ALT, and γ-glutamyltransferase (γ-GT) and longitudinally associated with lower type 2 diabetes risk (hazard ratio [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 (odds ratio [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 15 so-far-identified 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

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Received 4 June 2018 and accepted 29 September 2018
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db18-0620/-/DC1.

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 relationship 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), whereas 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 CpG sites 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 nonalcoholic fatty liver disease (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.

RESEARCH DESIGN AND METHODS

Study Population

The current 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 31 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 case subjects with incident type 2 diabetes that occurred until the censoring date (n = 820). In line with the case-cohort design, there was an overlap of 94 case subjects 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 case subjects with type 2 diabetes, 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 case subjects with incident type 2 diabetes were randomly selected (27). The following matching criteria were applied: age (±6 months), sex, fasting time (<3 h, 3 to <6 h, 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 control subject without diabetes, which was drawn from participants that had at least the same diabetes-free follow-up time of the respective case subject (incidence density sampling). Ten pairs were excluded from analyses because DNA samples did not pass quality control for methylation profiling.

Detection of Case Subjects With Incident Type 2 Diabetes

Systematic information sources for incident case subjects 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 case subjects with a diagnosis of type 2 diabetes

(ICD-10 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 (DY674; R&D systems), IGF-1 and IGFBP-3 (BioVendor Laboratorní medicína a.s., Brno, Czech Republic), and adiponectin (LINCO Research, St. Charles, MO); HDL cholesterol, triglycerides, glucose, HbA_{1c} 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. Genomic DNA (750 ng) from each participant was bisulfite-converted using Zymo EZ-96 DNA Methylation (Zymo Research Corp., Irvine, CA) and then hybridized on Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). The Illumina EPIC chip covered 890,703 cytosine positions located in transcription start site (TSS)200, TSS1500, 5′UTR, 1Exon, gene body, 3′UTR, 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 β-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 were corrected for cell type heterogeneity between samples by using champ.refbase (32). Probes annotated to contain single nucleotide polymorphisms were excluded. DNA methylation data of all CpG sites annotated to *IGFBP-2* gene were considered for analysis. Data from the ENCyclopedia Of DNA Elements (ENCODE) project (33) were used to identify hepatic H3K27ac, H3K4me3, H3K9ac histone marks in the *IGFBP-2* gene (Supplementary Data).

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, dropout, 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/day, 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 also included the FLI, according to Bedogni et al. (34), which was calculated based on waist circumference, BMI, and blood concentration of triglycerides and γ-GT. The fifth model was additionally adjusted for established type 2 diabetes–related biomarkers (adiponectin, fetuin A, triglycerides, ALT, CRP, γ-GT). Glucose and HbA_{1c} 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, βs 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) <0.05 was considered statistically significant. Where applicable, multiplicity of tests was considered by controlling the false discovery rate according to Hochberg and Benjamini (35).

RESULTS

Baseline Characteristics and Correlation Structure

Baseline characteristics of the study population are shown in Table 1. The median IGFBP-2 concentration was 92 ng/mL (interquartile range 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

Table 1—Baseline characteristics over IGFBP-2 quintile-based groups, EPIC-Potsdam Study (subcohort, *n* = 2,108)

	Subgroups according to quintiles of IGFBP-2					
	Q1* (*n* = 422)	Q2 (*n* = 421)	Q3 (*n* = 422)	Q4 (*n* = 421)	Q5 (*n* = 422)	All
IGFBP-2, ng/mL	41 (1–54)	65 (54–78)	92 (78–105)	121 (105–142)	175 (142–666)	92 (59, 129)
Sex, female %	67	63	62	60	60	62
Age at baseline, years	45 (40, 54)	48 (41, 57)	51 (43, 59)	52 (43, 59)	54 (44, 60)	50 (42, 58)
BMI, kg/m^2	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 use, 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)
Triglycerides, mg/dL	133 (92, 199)	119 (83, 180)	106 (77, 158)	98 (69, 137)	88 (64, 129)	107 (77, 161)
Fetuin A, μg/mL	288 (248, 335)	266 (227, 312)	263 (220, 299)	258 (223, 301)	250 (212, 290)	265 (226, 307)
ALT, units/L	22 (15, 37)	21 (15, 31)	20 (15, 28)	19 (14, 24)	17 (14, 22)	20 (15, 28)
γ-GT, units/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)
HbA_{1c}, %	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)
HbA_{1c}, 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)

Data are median (minimum–maximum), median (interquartile range), or %. *The representative subcohort was divided into five subgroups (Q1–Q5) according to quintiles of the distribution of IGFBP-2 plasma concentrations.

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.

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.

In restricted cubic spline analyses, a nonlinear 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.

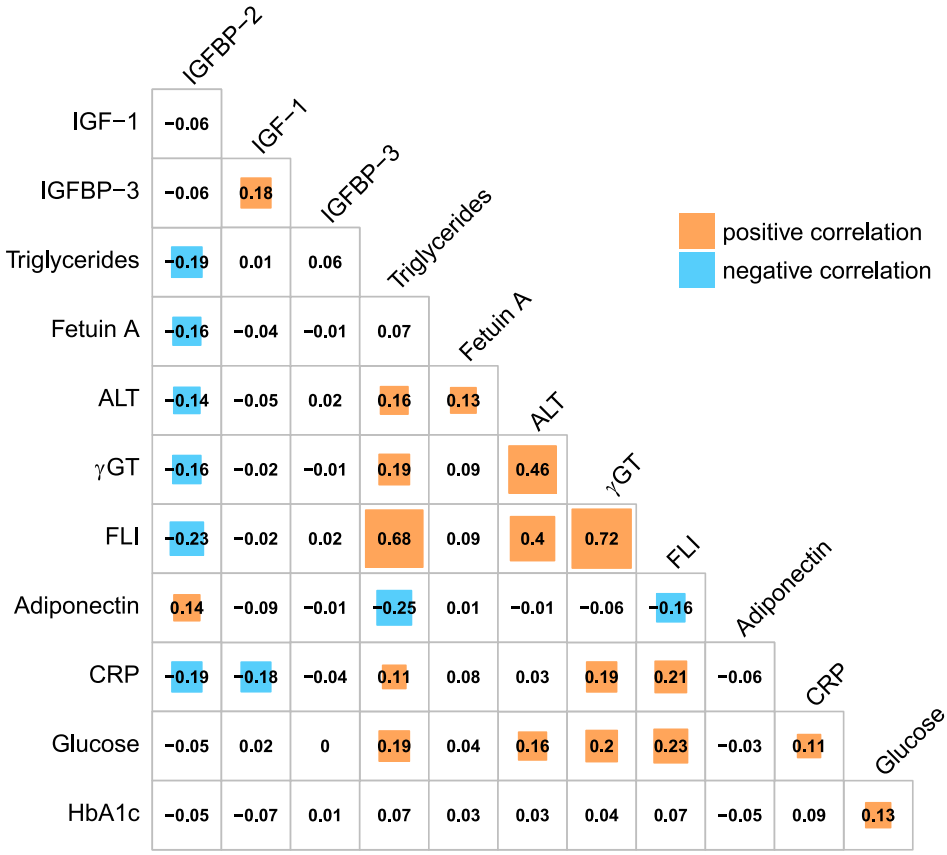

Figure 1—Correlation structure between IGFBP-2 and other type 2 diabetes–related biomarkers. Numbers indicate age-, sex-, and waist circumference–adjusted Spearman correlation coefficients. The size of the colored rectangles corresponds to the strength of correlations (complete color filling, absolute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

Regression diagnostics did not indicate violation of the proportional hazards assumption (Supplementary Figs. 2 and 3). Excluding participants for whom type 2 diabetes was diagnosed within the first 2 years of follow-up, excluding participants with high HbA_{1c} (≥5.7%), or restricting the analysis to nonfasted participants did not substantially change the results (data not shown).

DNA Methylation in *IGFBP-2* and Type 2 Diabetes Risk

Taking advantage of the high resolution of Illumina Human Methylation BeadChips, DNA methylation levels of 33 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 a low degree of DNA methylation (e.g., cg03625261, cg26187237, cg25316969) and those located 25 kb downstream the CpG island showed intermediate to high levels of methylation (Fig. 2).

Hypermethylated CpGs in case subjects were exclusively located in the promoter region in proximity to active histone marks, whereas two of three CpGs that were hypomethylated in case subjects were located in or close to the gene body (Fig. 2, Table 3). The link between epigenetic variation in the *IGFBP-2* gene and type 2 diabetes risk was summarized in a weighted *IGFBP-2* DNA methylation score. Higher methylation score points were associated with a substantially higher risk of diabetes incidence (OR per SD higher methylation score 2.7, 95% CI 2.1, 3.5) (Table 4). In cross-sectional analyses stratified by case status, the methylation score points were not associated with circulating IGFBP-2 concentrations (log-transformed). The standardized βs (*P* values) were 0.04 (0.1) and −0.01 (0.8) in case and control subjects, respectively, in a model adjusted for age, sex, and lifestyle variables. Adjustment for circulating IGFBP-2 concentration, waist circumference, FLI, and HbA_{1c}, and random glucose, respectively, did not attenuate the relationship between methylation score and type 2 diabetes risk (Table 4). Again, these results were highly robust in subanalyses restricted to participants with at least 2 years of type 2 diabetes–free survival time and HbA_{1c} levels <5.7% (data not shown).

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

	Pooled (n = 2,778, n_{cases} = 755)		Men (n = 1,188, n_{cases} = 444)		Women (n = 1,590, 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

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 IGFBP-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 HbA_{1c}. *HR of developing type 2 diabetes associated with 1 SD higher circulating IGFBP-2 levels.

DISCUSSION

We found strong inverse associations of circulating IGFBP-2 concentrations with type 2 diabetes risk in both sexes, which were robust against comprehensively controlling for established phenotypic and metabolic risk factors. For example, type 2 diabetes risk was more than doubled for participants with moderately low compared with participants with moderately high circulating IGFBP-2 concentration (1 SD below vs. 1 SD above the mean). Moreover, methylation levels of the *IGFBP-2* gene were also strongly linked to type 2 diabetes incidence. After accounting for various other risk factors, the odds of developing diabetes was more than six times higher for participants with a moderately high compared with those with a moderately low methylation score (1 SD below vs. 1 SD above the mean). To our knowledge, this is the first population-based prospective study on the relationship between IGFBP-2 and type 2 diabetes risk in both sexes and the first investigation of the longitudinal relation between *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. (7) observed strong and robust associations of IGFBP-2 with type 2 diabetes risk within the Nurses' Health Study (NHS). Women in the highest IGFBP-2 quintile-based group had a five times lower type 2 diabetes risk compared those in the lowest group. In our study, the comprehensively adjusted effect estimate for four SDs higher IGFBP-2 concentrations also translates into an approximately five times lower type 2 diabetes risk. Rajpathak et al. (7) raised the question as to 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 relationship 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

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 case subjects and n = 290 control subjects), nested within the EPIC-Potsdam cohort study. Control subjects were drawn by incidence density sampling and matched for age (±6 months), sex, fasting time (<3 h, 3 to <6 h, 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 case and control subjects after correcting for multiple testing. †OR of developing type 2 diabetes associated with 1 SD higher methylation adjusted for alcohol consumption, smoking, and physical activity as well as age and sex (matching variables).

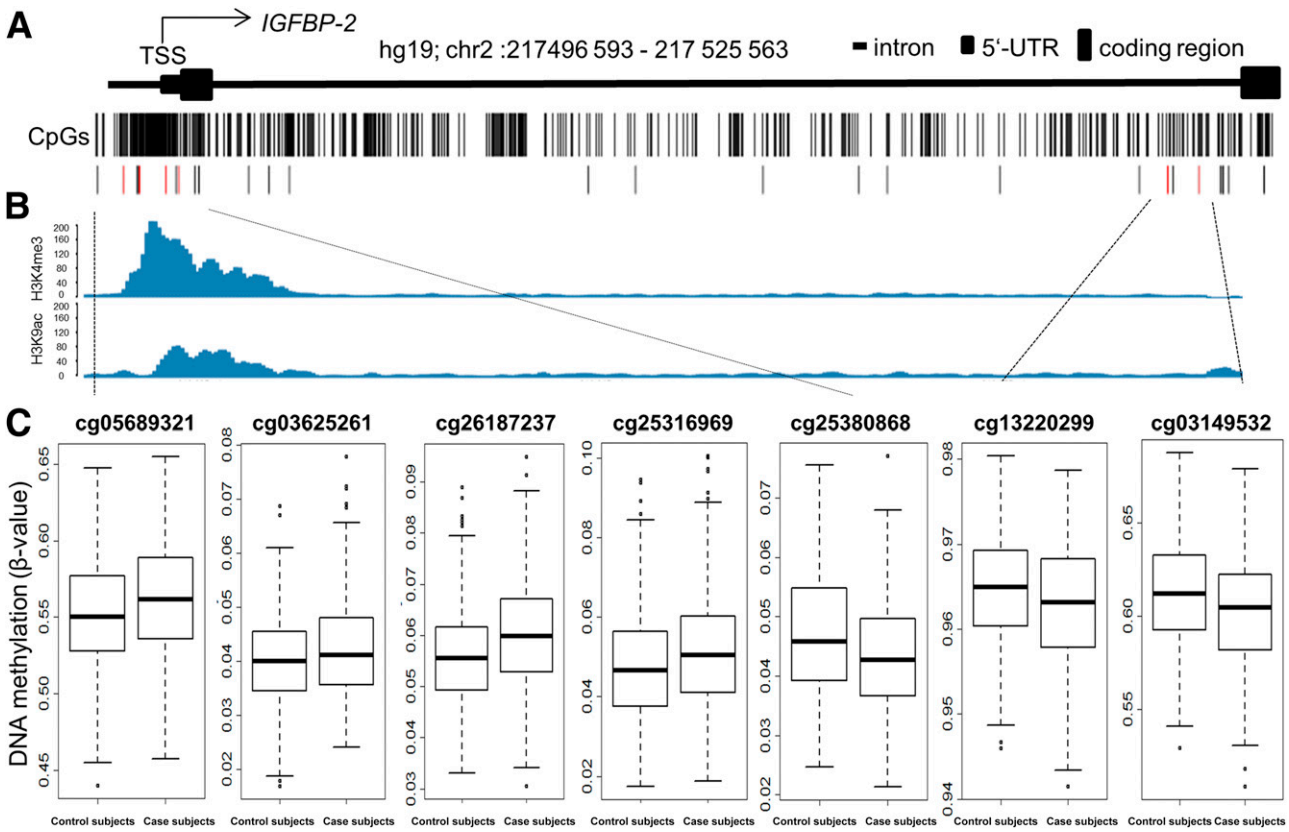

Figure 2—Differential DNA methylation levels of *IGFBP-2* gene in case and control subjects. *A*: Genomic organization of *IGFBP-2* gene 1,500 bp upstream of the TSS up to the end of exon 2. All CpG sites located in this region (spanning from chr2: 217496593 to 217525563) are indicated as lines in the upper part. Depicted in the lower part are CpGs covered by Illumina array (black), and CpGs significantly different between case and control subjects are highlighted in red. *B*: Hepatic pattern of active histone marks H3K4me3 and H3K9ac (ENCODE data). *C*: Boxplots of DNA methylation levels in case and control subjects for CpGs that were statistically significantly differentially methylated between the two groups after controlling the false discovery rate.

insulin sensitivity in young (8 and 16 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 constitutes a compensatory system under challenged conditions.

Under nonexperimental conditions, interpretation of the relationship 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 HbA_{1c} and fasting insulin (7). Consistently, adjustment for glucose parameters or exclusion of participants with elevated baseline-HbA_{1c} or with a type 2 diabetes diagnosis within the first 2 years of follow-up did not markedly attenuate 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 (7 out of 33) 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

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
	IGFBP-2	2.83	2.1, 3.81
	Waist circumference	3.15	2.25, 4.42
	FLI	3.25	2.25, 4.68
	HbA_{1c} 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 case subjects and n = 290 control subjects), nested within the EPIC-Potsdam cohort study. Control subjects were drawn by incidence density sampling and matched for age (±6 months), sex, fasting time (<3 h, 3 to <6 h, and ≥6 h before blood draw), time of day of blood sampling (±2 h), and season at blood sampling. *OR of developing type 2 diabetes associated with 1 SD higher methylation score; all models were adjusted for alcohol consumption, smoking, and physical activity as well as age and sex (matching variables).

a luciferase reporter construct of the homologous region in mice exhibits promoter activity (15). Taken together, hypermethylation of CpGs located in such an important *cis* regulatory region is likely to interact with regulatory transcription factors and may affect *IGFBP-2* expression.

Hypomethylation of two CpGs sites (cg03149532, cg13220299) located in the end of intron 2 was linked to higher type 2 diabetes risk. Recent studies suggested that low levels of DNA methylation within the gene body were related to inactive gene expression (40). The robust association of the methylation score in subanalyses excluding participants with an early diabetes diagnosis and with elevated HbA_{1c} levels makes reverse causation through undiagnosed type 2 diabetes unlikely. DNA methylation seems to be a stable marker. Thus, the observed link of methylation of *IGFBP-2* with diabetes risk suggests that epigenetic silencing of the *IGFBP-2* gene may predispose for the onset of the disease. Still, our findings on epigenetic alterations need to be interpreted with caution because we were the first to conduct a targeted investigation of DNA methylation in the *IGFBP-2* gene region and incident type 2 diabetes.

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

We further hypothesized that the effect of *IGFBP-2* DNA methylation on type 2 diabetes risk might be mediated by early impairment of insulin signaling and by facilitation of visceral adiposity and liver fat accumulation. However, conditioning the relationship between methylation score and type 2 diabetes risk on HbA_{1c} and glucose only slightly attenuated the risk estimate, and conditioning on waist circumference and FLI, respectively, resulted in stronger risk estimates. These results do not support our a priori mediation hypotheses. We can still not rule out the possibility that *IGFBP-2* DNA methylation may affect aspects of insulin sensitivity and hepatic lipid metabolism that are less well captured by the phenotypic markers that we used to reflect these physiological traits.

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

Our study had limitations. First, based on our observations we can only speculate on potential causal paths that link IGFBP-2 to type 2 diabetes development. We used a prospective cohort design, comprehensively adjusted for potential phenotypical and lifestyle confounders and a large set of known type 2 diabetes–related biomarkers, and our results were robust in subanalyses restricted to participants who remained free of type 2 diabetes for at least 2 years after recruitment and who did not have elevated HbA_{1c} levels at baseline. Thus, our results cannot be explained by confounding through known type 2 diabetes risk factors, and reverse causation should not be an issue. Now, conclusive animal experiments and Mendelian randomization studies may be applicable to elucidate the mechanisms that implicate IGFBP-2 in type 2 diabetes development and to clarify whether IGFBP-2 itself causally affects type 2 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.

Acknowledgments. The authors thank all participants of the EPIC-Potsdam study. Furthermore, they acknowledge the Human Study Centre (HSC) of the German Institute of Human Nutrition Potsdam-Rehbrücke for providing the data sets, in particular, Dr. Manuela Bergmann, the leader of HSC, for supervising the process of data generation and Ellen Kohlsdorf for data management.

Funding. The study was supported by the Federal Ministry of Science, Germany (grant 01 EA 9401) and the European Union (grant SOC 95201408 05 F02) for the recruitment phase of the EPIC-Potsdam Study, by the German Cancer Aid (grant 70-2488-Ha I) and the European Community (grant SOC 98200769 05 F02) for the follow-up of the EPIC-Potsdam Study, and by a grant from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD) (82DZD00302) and the State of Brandenburg.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. C.W., M.O., M.B.S., and A.S. developed the project, designed the analysis plan, wrote the manuscript, and had primary responsibility for the final content. C.W., M.O., O.K., M.J., and P.G. conducted statistical analyses. A.T., J.K., H.G., T.P., and H.B. contributed to the acquisition of data. C.W., M.O., K.M., T.P., M.B.S., and A.S. contributed to the interpretation of data. All authors contributed to revising the manuscript for critically for important intellectual content and read and approved the final version of the manuscript. A.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Moses AC, Young SC, Morrow LA, O'Brien M, Clemmons DR. Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. Diabetes 1996;45:91–100
2. Scavo LM, Karas M, Murray M, Leroith D. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. J Clin Endocrinol Metab 2004;89:3543–3553
3. Di Cola G, Cool MH, Accili D. Hypoglycemic effect of insulin-like growth factor-1 in mice lacking insulin receptors. J Clin Invest 1997;99:2538–2544.
4. Sandhu MS, Heald AH, Gibson JM, Cruickshank JK, Dunger DB, Wareham NJ. Circulating concentrations of insulin-like growth factor-I and development of glucose intolerance: a prospective observational study. Lancet 2002;359:1740–1745
5. Lewitt MS, Hilding A, Brismar K, Efendic S, Ostenson CG, Hall K. IGF-binding protein 1 and abdominal obesity in the development of type 2 diabetes in women. Eur J Endocrinol 2010;163:233–242
6. Lewitt MS, Hilding A, Ostenson CG, Efendic S, Brismar K, Hall K. Insulin-like growth factor-binding protein-1 in the prediction and development of type 2 diabetes in middle-aged Swedish men. Diabetologia 2008;51:1135–1145
7. Rajpathak SN, He M, Sun Q, et al. Insulin-like growth factor axis and risk of type 2 diabetes in women. Diabetes 2012;61:2248–2254
8. Drogan D, Schulze MB, Boeing H, Pischon T. Insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 in relation to the risk of type 2 diabetes mellitus: results from the EPIC-Potsdam Study. Am J Epidemiol 2016;183:553–560
9. Russo VC, Azar WJ, Yau SW, Sabin MA, Werther GA. IGFBP-2: The dark horse in metabolism and cancer. Cytokine Growth Factor Rev 2015;26:329–346
10. Li Z, Wu Z, Ren G, Zhao Y, Liu D. Expression patterns of insulin-like growth factor system members and their correlations with growth and carcass traits in Landrace and Lantang pigs during postnatal development. Mol Biol Rep 2013;40:3569–3576
11. Assefa B, Mahmoud AM, Pfeiffer AFH, Birkenfeld AL, Spranger J, Arafat AM. Insulin-like growth factor (IGF) binding protein-2, independently of IGF-1, induces GLUT-4 translocation and glucose uptake in 3T3-L1 adipocytes. Oxid Med Cell Longev 2017;2017:3035184
12. Wheatcroft SB, Kearney MT, Shah AM, et al. IGF-binding protein-2 protects against the development of obesity and insulin resistance. Diabetes 2007;56:285–294
13. Hedbacker K, Birsoy K, Wysocki RW, et al. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metab 2010;11:11–22
14. Sabin MA, Russo VC, Azar WJ, Yau SW, Kiess W, Werther GA. IGFBP-2 at the interface of growth and metabolism–implications for childhood obesity. Pediatr Endocrinol Rev 2011;8:382–393
15. Kammel A, Saussenthaler S, Jähnert M, et al. Early hypermethylation of hepatic Igfbp2 results in its reduced expression preceding fatty liver in mice. Hum Mol Genet 2016;25:2588–2599
16. Ahrens M, Ammerpohl O, von Schönfels W, et al. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. Cell Metab 2013;18:296–302
17. Fradin D, Bougnères P. T2DM: why epigenetics? J Nutr Metab 2011;2011:647514
18. Davegårdh C, García-Calzón S, Bacos K, Ling C. DNA methylation in the pathogenesis of type 2 diabetes in humans. Mol Metab 2018;14:12–25
19. Ouni M, Gunes Y, Belot MP, Castell AL, Fradin D, Bougnères P. The IGF1 P2 promoter is an epigenetic QTL for circulating IGF1 and human growth. Clin Epigenetics 2015;7:22
20. Ouni M, Belot MP, Castell AL, Fradin D, Bougnères P. The P2 promoter of the IGF1 gene is a major epigenetic locus for GH responsiveness. Pharmacogenomics J 2016;16:102–106
21. Vu TH, Hoffman AR. Promoter-specific imprinting of the human insulin-like growth factor-II gene. Nature 1994;371:714–717
22. Gu T, Gu HF, Hilding A, et al. Increased DNA methylation levels of the insulin-like growth factor binding protein 1 gene are associated with type 2 diabetes in Swedish men. Clin Epigenetics 2013;5:21
23. Siqueira FR, Furukawa LN, Oliveira IB, Heimann JC. Glucose metabolism and hepatic Igf1 DNA methylation are altered in the offspring of dams fed a low-salt diet during pregnancy. Physiol Behav 2016;154:68–75
24. Desgagné V, Hivert MF, St-Pierre J, et al. Epigenetic dysregulation of the IGF system in placenta of newborns exposed to maternal impaired glucose tolerance. Epigenomics 2014;6:193–207
25. Boeing H, Korfmann A, Bergmann MM. Recruitment procedures of EPIC-Germany. European Investigation into Cancer and Nutrition. Ann Nutr Metab 1999;43:205–215
26. Schienkiewitz A, Schulze MB, Hoffmann K, Kroke A, Boeing H. Body mass index history and risk of type 2 diabetes: results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Am J Clin Nutr 2006;84:427–433
27. Drogan D, Dunn WB, Lin W, et al. Untargeted metabolic profiling identifies altered serum metabolites of type 2 diabetes mellitus in a prospective, nested case control study. Clin Chem 2015;61:487–497
28. Boeing H, Wahrendorf J, Becker N. EPIC-Germany–A source for studies into diet and risk of chronic diseases. European Investigation into Cancer and Nutrition. Ann Nutr Metab 1999;43:195–204
29. Enzenbach C, Kröger J, Zietemann V, et al. Erythrocyte membrane phospholipid polyunsaturated fatty acids are related to plasma C-reactive protein and adiponectin in middle-aged German women and men. Eur J Nutr 2011;50:625–636
30. Morris TJ, Beck S. Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. Methods 2015;72:3–8
31. Butcher LM, Beck S. Probe Lasso: a novel method to rope in differentially methylated regions with 450K DNA methylation data. Methods 2015;72:21–28

32. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 2012;13:86
33. Sloan CA, Chan ET, Davidson JM, et al. ENCODE data at the ENCODE portal. Nucleic Acids Res 2016;44(D1):D726–D732
34. Bedogni G, Bellentani S, Miglioli L, et al. The fatty liver index: a simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol 2006;6:33
35. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. Stat Med 1990;9:811–818
36. DeMambro VE, Clemmons DR, Horton LG, et al. Gender-specific changes in bone turnover and skeletal architecture in igfbp-2-null mice. Endocrinology 2008; 149:2051–2061
37. Frystyk J. Free insulin-like growth factors–measurements and relationships to growth hormone secretion and glucose homeostasis. Growth Horm IGF Res 2004;14:337–375
38. Heald AH, Kaushal K, Siddals KW, Rudenski AS, Anderson SG, Gibson JM. Insulin-like growth factor binding protein-2 (IGFBP-2) is a marker for the metabolic syndrome. Exp Clin Endocrinol Diabetes 2006;114:371–376
39. Martin RM, Holly JM, Davey Smith G, Gunnell D. Associations of adiposity from childhood into adulthood with insulin resistance and the insulin-like growth factor system: 65-year follow-up of the Boyd Orr Cohort. J Clin Endocrinol Metab 2006;91:3287–3295
40. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–492