Influence of Polymorphisms in Genes Encoding for Insulin-Like Growth Factor (IGF)-I, Insulin, and IGF-Binding Protein (IGFBP)-3 on IGF-I, IGF-II, and IGFBP-3 Levels in Umbilical Cord Plasma

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Key Words

Polymorphism · IGF-I · IGF-II · IGFBP-3 · Umbilical cord plasma

Abstract

Background/Aims: In postnatal life, polymorphisms in the promoter region of *IGFBP3* were associated with insulin-like growth factor binding protein (IGFBP)-3 plasma levels. Whether these associations exist in utero has not been studied yet. Polymorphisms in the *IGF1* promoter (polymorphic CA-repeat) and the insulin gene variable number tandem repeats locus (*INS VNTR*) are further polymorphisms of interest, because associations with birth weight have been reported. We aimed to investigate associations between polymorphisms in the promoter regions of *IGF1* (wild type 192 bp), *IGFBP3* (rs2854744; rs13241830), and *INS VNTR* (rs689) with cord plasma levels of IGF-I, IGF-II, and IGFBP-3. **Methods:** We measured IGF-I, IGF-II, and IGFBP-3 concentrations in cord blood from 677 neonates and genotyped the selected polymorphisms. **Results:** Carriers of the minor allele of both polymorphisms in the *IGFBP3* gene had, on average, 4–5% lower

IGFBP-3 levels per copy of the respective minor allele ($p = 0.002$ and $p = 0.028$) when compared to wild type carriers. The *IGF1* promoter and the *INS VNTR* polymorphisms were not associated with IGF-I, IGF-II, or IGFBP-3 levels. **Conclusions:** Our data show associations of cord plasma IGFBP-3 levels and the *IGFBP3* gene variants but not of *IGF1* promoter and *INS VNTR* polymorphisms with IGF-I, IGF-II, or IGFBP-3 levels in utero.

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Introduction

In fetal life, insulin, insulin-like growth factor (IGF)-I, IGF-II, and IGF-binding protein (IGFBP)-3 are essential growth factors [1–3]. Until now, however, the influence of common genetic variations on the in utero regulation of these growth factors has been studied only scarcely.

With respect to genetic influences of IGFBP-3 synthesis in utero, two polymorphisms in the promoter region of the *IGFBP3* gene (–202 A/C, rs2854744 and –185 C/T, rs13241830) are of particular interest; in vitro, the –202

A/C and the -185 C/T polymorphisms were recently shown to influence transcriptional activity of the *IGFBP3* gene in a haplotype-dependent way [4]. In several studies, the -202 A/C polymorphism was consistently shown to be associated with circulating levels of IGFBP-3. Until now, these associations have only been reported in adult [5–10] and pediatric populations [11, 12].

However, regulation of circulating levels of IGFs and their binding proteins varies considerably throughout life. While growth hormone is the predominant stimulator of IGF-I and IGFBP-3 synthesis in postnatal life, fetal production mainly depends on nutrient supply of the fetus [1]. It is thus of interest whether the association reported in postnatal life also exists in utero when different regulatory mechanisms of IGF and IGFBP production occur. Verifying a robust association would affirm the independent effects of this genetic variation on IGFBP-3 levels and add information on molecular influences on the regulation of fetal growth factor synthesis.

Common genetic variation has been suspected to influence the intrauterine regulation of insulin, IGF-I, and IGF-II synthesis. A polymorphic CA-repeat (wild type 192 bp) in the promoter region of the *IGF1* gene and an allele length variation at the variable number of tandem repeats (VNTR) locus in the promoter region of the insulin gene (*INS*; rs689) are interesting candidates because associations with birth weight have been described [13, 14], which were not confirmed by all studies [15–18]. In vitro, the *INS* VNTR polymorphism was shown to not only affect the expression of *INS* [19, 20] but also the expression of the nearby gene encoding for IGF-II [21].

Elucidating the influence of both polymorphisms on the in utero synthesis of IGFs and their binding proteins is of particular interest, since these polymorphisms have been hypothesized to explain the association between restricted fetal growth and an increased risk of diabetes and cardiovascular disease in later life, as reported in several population studies [22–24]. While insulin affects fetal growth and plays a crucial role in glucose homeostasis, IGF-I is also involved in glucose homeostasis [25, 26] and has been shown to be associated with cardiovascular disease [27–29]. If the specified polymorphisms affected circulating levels of IGFs and their binding proteins in utero, these associations would support their role in fetal growth and later susceptibility to diseases such as cardiovascular disease.

The purpose of this study was to investigate the relationship between the specified polymorphisms in the promoter region of the *IGF1*, the *IGFBP3*, and the insulin gene and their influence on concentrations of IGF-I,

IGF-II, and IGFBP-3 in umbilical cord plasma. Thereby, we aimed to gain deeper insights into the influence of common genetic variations on fetal IGF and IGFBP synthesis.

Subjects, Materials, and Methods

Study Population

Umbilical cord blood was prospectively collected from all neonates born between October 2004 and October 2006 at the Department of Obstetrics and Gynecology, Justus Liebig University of Giessen, Germany, and at the Department of Obstetrics and Gynecology, Asklepios Hospital, Lich, Germany, whose parents gave written informed consent for their child to participate in the study.

Immediately after delivery, umbilical cord blood samples were taken by the midwives. The samples were kept at 4 °C for up to 24 h before centrifugation at 3,000 g for 3 min. Cord plasma and cell pellet were separated and stored at -20 °C until analyzed.

Routine measurements of birth weight and length were registered and information about the pregnancy was taken from data documented at the prenatal visits in a standardized way. Information on further specific variables with theoretical or previously reported influence on fetal growth, such as parental weight and height, maternal smoking during pregnancy, and parental education, were obtained by interview. The calculation of gestational age was based on maternal menstrual history or on early prenatal ultrasound. Neonates of a gestational age <37 weeks were considered as born preterm. Being born extremely small for gestational age (ESGA), small for gestational age (SGA), appropriate for gestational age (AGA), or large for gestational age (LGA) was defined as a birth weight <3rd, 3rd to <10th, 10th to <90th, and ≥90th gender-specific percentile for German newborns [30], respectively. Maternal body mass index (BMI) was calculated by dividing the maternal prepregnancy weight (kg) by the squared maternal length (m). The variable hypertension was defined when systolic blood pressure values ≥140 mm Hg and/or diastolic blood pressure values ≥90 mm Hg were documented at the prenatal visits. The variable 'pregnancy weight gain' was calculated by subtracting maternal prepregnancy weight from maternal weight before delivery. The variable 'assisted reproduction' was defined as either in vitro fertilization or intracytoplasmic sperm injection. Maternal diabetes was defined as either type 1 diabetes or gestational diabetes with need for insulin therapy. All mothers who stated having smoked during pregnancy were classified as 'smokers'. Exclusion criteria were multiple births, unclear gestational age and, in the case of the neonates, suspected congenital infection syndromes, hemodynamically relevant cardiac malformations, identifiable syndromes, inborn endocrine diseases, and errors of metabolism. The analyses were confined to Caucasian neonates.

The study was approved by the Ethics Committee of the Justus Liebig University, Giessen.

Genotyping

DNA Isolation and Quantification. Genomic DNA without prior cell sorting as has been described by others [e.g. 31, 32] was purified from buffy coats of umbilical cord blood samples using the QIAamp® DNA Blood Mini kit (Qiagen GmbH, Hilden, Ger-

many). DNA was quantified using the human DNA Quantifiler® kit (P/N 4343895, Applied Biosystems, Foster City, Calif., USA).

INS VNTR or HphI -23 A/T (rs689). DNA samples were genotyped for the class I/III INS VNTR polymorphism at the -23 A/T HphI site located at nucleotide 4462 on GenBank sequence L15440. This polymorphism is in virtually complete linkage disequilibrium with the INS VNTR polymorphism [33]. The region of the promoter of the insulin gene was amplified with PCR using an SNP genotyping assay (5'>nuclease assay). Primers were supplied by MWG Biotech (Ebersberg, Germany) with the forward primer being 5'-CCATGGCAGAAGGACAGTGA-3' and the reverse primer 5'-GGGCACCTGGCCTTCAG-3'. Fluorescence and minor groove binder (MGB) labeled probes (Applied Biosystems, Warrington, UK) were used to discriminate for the two allelic variations: 6FAM-CCTGCCTGTCTCCCAGA-MGB and VIC-CTGCCTGTACCCAGA-MGB. PCR amplification was performed in 25 µl volume containing 20 ng genomic DNA, 10 pmol/l of each primer, 3 pmol/l of each probe, and TaqMan® Universal Master Mix containing buffer and AmpliTaq Gold® DNA polymerase (Applied Biosystems). After a pre-read run at 50°C to determine baseline fluorescence, amplification was performed at the following PCR conditions: 50°C for 2 min and 95°C for 10 min as an initial step, followed by 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min on an ABI 7500 real-time PCR system (Applied Biosystems). A post-read at 60°C was carried out to get an allelic discrimination.

IGFBP3 -185 C/T (rs13241830). DNA was genotyped for IGFBP3 -185 C/T polymorphism using a TaqMan® Pre-Designed SNP Genotyping Assay (C_1842666_10, Applied Biosystems) according to the manufacturer's instructions. A mix of primer and probes was supplied, the latter being 5'-fluorescence and 3'-MGB labeled. Reagents and PCR conditions were used as described above.

IGFBP3 -202 A/C (rs2854744). Genotyping was performed using 5'-CACCTTGGTTCTTGTAACGACAA-3' as forward primer and 5'-GGCGTGCAGCTCGAGACT-3' as reverse primer. TaqMan probes were FAM-CTCGTGCTCACGCC-MGB and VIC-TCCTCGTGCGCACG-MGB. Universal master mix and PCR conditions were used as described above. All SNPs were run in duplicate; concordance for replicate samples was 100%.

IGF-I Microsatellite Length Polymorphism. The microsatellite length polymorphism in the promoter region of the IGF1 gene was determined by fragment length analysis as described previously [34].

Biochemical Analyses

IGF-I and IGFBP-3 concentrations were measured using a radioimmunoassay (RIA) as described previously by Blum and Ranke [35]. For IGF-I, intra- and interassay coefficients of variation were 1.6 and 6.4%, respectively. Sensitivity was 0.3 µg/l under standard conditions. For IGFBP-3, intra- and interassay coefficients of variation were 1.9 and 9.2%, respectively, with a sensitivity of 0.13 mg/l under standard conditions.

IGF-II concentrations were measured by RIA using a commercially available assay (Mediagnost, Tübingen, Germany). All samples were run in duplicate.

Statistical Analyses

To compare characteristics of neonates born at term and neonates born preterm we applied unpaired t tests, nonparametric Wilcoxon tests, Pearson's χ^2 test ANOVA, and unpaired Kruskal-

Wallis test where appropriate. Pearson's correlation coefficient (r) and Spearman's ρ (r_s) were used to calculate the correlations between continuous variables with normal and non-normal distributions as appropriate, and Spearman's ρ (r_s) was used for correlation analysis between continuous and categorical variables. Genotype and allele frequencies were estimated, and a test of Hardy-Weinberg equilibrium was performed using the asymptotic χ^2 test. Linkage disequilibrium between the -202 A/C and the -185 C/T polymorphism was assessed by the correlation coefficient r and Lewontin's D' . Haplotypes based on the two SNPs from IGFBP3 were estimated via the expectation maximization algorithm by the SAS procedure PROC HAPLOTYPE (version 9.1, release 2004).

General linear regression models of various adjustments were designed to assess the influence of the investigated polymorphisms on the cord plasma levels of IGF-I, IGF-II, and IGFBP-3. An additive genetic model, i.e. a model estimating the change of mean IGF-I, IGF-II, and IGFBP-3 levels per copy of the minor allele, was applied. The covariate 'birth weight for gestational age' as an indicator of pathological intrauterine growth was categorized as ESGA, SGA, AGA, and LGA and alternatively as categorization of gender-specific quintiles derived from the studied population. An additional analysis for IGFBP-3 included the haplotypes instead of single SNPs to the model. All models were run including (a) all children and (b) only the children born at term.

All analyses were conducted in SPSS version 18.0 software (SPSS Inc., Chicago, Ill., USA). Statistical significance of differences was assumed if p was <0.05 .

Results

A total of 754 parents consented to enroll their children in the study. Among those children, 77 did not fulfill inclusion criteria (at least one parent being non-Caucasian: $n = 22$; twin birth: $n = 30$; unclear gestational age, suspected congenital infection syndromes, hemodynamically relevant cardiac malformation, identifiable syndrome, endocrine disease, or inborn error of metabolism: $n = 25$). Thus, 677 neonates were included in the study. Basic maternal and infant characteristics are presented in table 1 for all 677 neonates and stratified for neonates born at term and those born preterm.

Biochemical Analyses

For all neonates, mean cord plasma concentrations of IGF-I, IGF-II, and IGFBP-3 were 59.8 ± 30.0 µg/l, 407 ± 137 µg/l, and 1.40 ± 0.46 mg/l, respectively. IGF-I concentrations were higher in female than in male neonates (64.2 ± 32 vs. 55.6 ± 28 µg/l; $p = 0.0002$) and increased with increasing birth weight for gestational age, as indicated by the categories ESGA, SGA, AGA, and LGA ($p < 0.0001$; table 2), as well as by the gender-specific quintiles

Table 1. Maternal and neonate characteristics of 677 pregnancies

Variable	All births (n = 677)	Neonates born at term (n = 555)	Neonates born preterm (n = 122)	p ^b
ESGA/SGA/AGA/LGA, %	2/8/77/13	2/8/78/12	3/8/73/16	0.50
Mean maternal age $\pm$ SD, years	30.6 $\pm$ 6	30.7 $\pm$ 6	30.3 $\pm$ 6	0.52
Primipara, %	39	37	48	0.0234
Smoking during pregnancy, % ^a	13	12	19	0.05
Mean gestational age in completed weeks $\pm$ SD	38.2 $\pm$ 2.6	39.2 $\pm$ 1.2	33.7 $\pm$ 2.5	<0.0001
Median	39	39	34	
Range	26–42	37–42	26–36	
Mean birth weight $\pm$ SD, g	3,243 $\pm$ 653	3,445 $\pm$ 447	2,327 $\pm$ 656	<0.0001
Median	3,340	3,430	2,405	
Range	750–4,910	1,790–4,910	750–4,170	
Male/female, %	51/49	51/49	53/47	0.62

^a Ever smoked during pregnancy on a regular basis. ^b p value for comparing neonates born at term and neonates born preterm.

Table 2. Cord plasma concentrations of IGF-I, IGF-II, and IGFBP-3 within the categories ESGA, SGA, AGA, and LGA

	ESGA (n = 12)	SGA (n = 54)	AGA (n = 523)	LGA (n = 88)	p value
IGF-I, μ g/l	22.5 $\pm$ 16.9	36.9 $\pm$ 20.7	59.6 $\pm$ 27.6	79.7 $\pm$ 34.6	<0.0001
IGF-II, μ g/l	411 $\pm$ 152	420 $\pm$ 160	408 $\pm$ 139	398 $\pm$ 108	0.84
IGFBP-3, mg/l	0.99 $\pm$ 0.24	1.20 $\pm$ 0.43	1.39 $\pm$ 0.45	1.59 $\pm$ 0.47	<0.0001

Concentrations are provided as means $\pm$ SD.

Table 3. Cord plasma concentrations of IGF-I, IGF-II, and IGFBP-3 within gender-specific quintiles of birth weight for gestational age – boys

	Quintile 1 (n = 62)	Quintile 2 (n = 75)	Quintile 3 (n = 71)	Quintile 4 (n = 73)	Quintile 5 (n = 65)	p value
IGF-I, μ g/l	37.0 $\pm$ 18.7	47.5 $\pm$ 20.2	55.5 $\pm$ 26.9	65.9 $\pm$ 24.3	71.6 $\pm$ 33.4	<0.0001
IGF-II, μ g/l	397 $\pm$ 138	413 $\pm$ 154	418 $\pm$ 154	423 $\pm$ 155	402 $\pm$ 115	0.83
IGFBP-3, mg/l	1.16 $\pm$ 0.38	1.36 $\pm$ 0.45	1.29 $\pm$ 0.35	1.47 $\pm$ 0.50	1.41 $\pm$ 0.44	<0.0001

Concentrations are provided as mean $\pm$ SD.

of birth weight of the studied population ($p < 0.0001$; tables 3, 4).

IGF-II concentrations did not differ between female and male neonates (404.4 ± 129 vs. 411.2 ± 144 μ g/l; $p = 0.52$) and were not influenced by birth weight for gestational age (table 2) or gender-specific quintiles of birth weight (tables 3, 4). IGFBP-3 concentrations were higher

in female than in male neonates (1.45 ± 0.48 vs. 1.34 ± 0.44 mg/l; $p = 0.0026$) and correlated positively with IGF-I levels ($r = 0.41$; $p < 0.0001$) and with gestational age ($r_s = 0.11$; $p = 0.003$). IGFBP-3 concentrations increased with increasing birth weight for gestational age ($p < 0.0001$; table 2) and across the gender-specific quintiles ($p < 0.0001$; tables 3, 4).

Table 4. Cord plasma concentrations of IGF-I, IGF-II, and IGFBP-3 within gender-specific quintiles of birth weight for gestational age – girls

	Quintile 1 (n = 62)	Quintile 2 (n = 67)	Quintile 3 (n = 69)	Quintile 4 (n = 68)	Quintile 5 (n = 63)	p value
IGF-I, µg/l	42.0 ± 25.6	56.6 ± 29.1	68.0 ± 30.7	68.0 ± 24.9	85.8 ± 31.2	<0.0001
IGF-II, µg/l	408 ± 151	410 ± 138	391 ± 106	399 ± 133	415 ± 115	0.83
IGFBP-3, mg/l	1.23 ± 0.36	1.40 ± 0.46	1.41 ± 0.40	1.53 ± 0.50	1.68 ± 0.53	<0.0001

Concentrations are provided as mean ± SD.

Table 5. Genotype frequency distributions in the study population

Polymorphism	Minor allele frequency			
IGFBP-3 A/C	AA 138 (20.5%)	CA 343 (51.0%)	CC 191 (28.4%)	54% ^a
IGFBP-3 C/T	CC 312 (46.6%)	CT 289 (43.2%)	TT 68 (10.2%)	32%
INS VNTR	class I/I 344 (51.1%)	class I/III 269 (40.0%)	class III/III 60 (8.9%)	29%
IGF-I 192 bp	192/192 264 (39.1%)	192/x 315 (46.7%)	x/x 96 (14.2%)	38% ^b

^a The C allele is designated as 'minor allele' in order to be consistent with previous studies [e.g. 9].

^b Non-carrying of the 192 bp allele is designated as 'minor allele'.

Genotyping Results

Genotype distributions of all four polymorphisms are shown in table 5. For the *IGF1* promoter polymorphism, nine different alleles were identified, the most frequent being the 192bp allele. For all polymorphisms, no significant differences across the three genotypes were found with respect to gender and the percentages of children born ESGA, SGA, AGA, or LGA. The genotype distributions were comparable to previously reported distributions [13, 16, 17] and were in Hardy-Weinberg equilibrium ($p > 0.50$). The minor allele frequencies for the polymorphisms ranged between 0.29 and 0.54. Lewontin's D' of the -185 and -202 polymorphisms of *IGFBP3* was almost unity ($D' = 0.9934$) with a correlation coefficient of $r = 0.63$.

Associations between Genotypes and Cord Blood Levels of IGF-I, IGF-II, and IGFBP-3

Associations of the polymorphisms in the promoter region of the *IGFBP3* gene with IGFBP-3 levels were calculated in a linear regression model. The variables gender,

gestational age, IGF-I, and birth weight for gestational age were included in the model. In order to identify further variables for inclusion in the linear regression model we chose the following approach: at first, correlations between the concentrations of IGF-I and IGFBP-3 and further variables with a theoretical or previously reported influence on birth weight (i.e. parity, maternal hypertension, assisted reproduction, maternal pregnancy weight gain, maternal BMI, maternal diabetes, and smoking during pregnancy) were calculated. Among these variables, the variables parity, maternal hypertension, weight gain during pregnancy, maternal diabetes, and smoking during pregnancy were shown to be correlated with IGF-I concentrations ($p < 0.05$). The variable maternal BMI was shown to be correlated with IGFBP-3 concentrations ($p = 0.028$). In a second step, all these variables which were shown to be correlated with either IGF-I or to IGFBP-3 concentrations were included in the model.

Both polymorphisms in the promoter region of the *IGFBP3* gene were significantly associated with IGFBP-3

Table 6. Mean plasma IGFBP-3 concentrations (mg/l) by genotypes in all neonates and in neonates born at term

Polymorphism		Genotype			p ^b
		AA	CA	CC	
IGFBP-3 -202 A/C polymorphism	IGFBP-3 ^a in all neonates	1.48 (1.41; 1.55) (n = 129)	1.40 (1.35; 1.44) (n = 319)	1.33 (1.28; 1.39) (n = 181)	0.002 ^c
	IGFBP-3 ^a in neonates born at term	1.51 (1.44; 1.59) (n=109)	1.45 (1.40; 1.50) (n=259)	1.34 (1.27; 1.40) (n=145)	0.0004
Polymorphism		Genotype			p ^b
		CC	CT	TT	
IGFBP-3 -185 C/T polymorphism	IGFBP-3 ^a in all neonates	1.43 (1.38; 1.48) (n = 293)	1.39 (1.34; 1.43) (n = 267)	1.31 (1.22; 1.41) (n = 66)	0.028 ^d
	IGFBP-3 ^a in neonates born at term	1.48 (1.43; 1.53) (n = 240)	1.42 (1.37; 1.48) (n = 218)	1.29 (1.18; 1.40) (n = 52)	0.003

^a Values are means and 95% CI [adjusted for gender, gestational age, IGF-I, birth weight for gestational age (categories ESGA, SGA, AGA, and LGA)], maternal BMI, parity hypertension, pregnancy weight gain, maternal diabetes, and smoking during pregnancy). ^b p value for additive models. ^c Regression formula: IGFBP-3 mg/l = $-0.271 - 0.073 \times \text{gender} [\text{male} = 1] + 0.028 \times \text{gestational age} [\text{week}] + 0.111 \times \text{weight for gestational age} [\text{categorized as ESGA/SGA/AGA/LGA}] + 0.005 \times \text{IGF-I} [\mu\text{g/l}] + 0.003 \times \text{BMI} [\text{kg/m}^2] - 0.008 \times \text{parity} + 0.027 \times \text{hypertension} [\text{yes} = 1] - 0.001 \times \text{weight gain}$

in pregnancy [kg] $- 0.41 \times \text{maternal diabetes} [\text{yes} = 1] + 0.099 \times \text{smoking during pregnancy} [\text{yes} = 1]$. ^d IGFBP-3 mg/l = $-0.333 - 0.054 \times \text{each copy of the minor allele of the IGFBP-3 gene} - 0.64 \times \text{gender} [\text{male} = 1] + 0.029 \times \text{gestational age} [\text{week}] + 0.105 \times \text{weight for gestational age} [\text{categorized as ESGA/SGA/AGA/LGA}] + 0.005 \times \text{IGF-I} [\mu\text{g/l}] + 0.003 \times \text{BMI} [\text{kg/m}^2] - 0.009 \times \text{parity} + 0.043 \times \text{hypertension} [\text{yes} = 1] - 0.001 \times \text{weight gain in pregnancy} [\text{kg}] - 0.051 \times \text{maternal diabetes} [\text{yes} = 1] + 0.089 \times \text{smoking during pregnancy} [\text{yes} = 1]$.

levels (table 6). The -202 A/C polymorphism of *IGFBP3* showed a clear additive association per copy of the C allele with mean IGFBP-3 concentrations of 1.48, 1.40, and 1.33 mg/l for 0, 1, and 2 copies of the C allele, respectively ($p = 0.002$). In this linear regression model, each copy of the minor allele decreased the IGFBP-3 levels adjusted for gender, gestational age, IGF-I, 'birth weight for gestational age', parity, maternal BMI, maternal hypertension, weight gain during pregnancy, maternal diabetes, and smoking during pregnancy by 0.073 mg/l on average, which translates to a relative decrease of 5%. This association was slightly more pronounced when the analyses were confined to neonates born at term ($p = 0.0004$). We observed similar results irrespective of whether we adjusted for 'birth weight for gestational age' categorized by the gender-specific quintiles or classified as ESGA, SGA, AGA, or LGA. Very similar results were obtained for the -185 C/T polymorphism of *IGFBP3* with an average decrease of each copy of the T allele by 0.054 mg/l ($p = 0.028$; table 6). Again, this association was more pronounced when the analyses were confined to neonates born at term ($p = 0.003$).

Three common (AC, CC, and CT) and one rare haplotype (AT) derived from these two polymorphisms were identified. The rare haplotype was not analyzed in the models. Consistent with the single polymorphism analysis, individuals carrying two copies of the haplotype AC had the highest mean IGFBP-3 concentrations when compared to those carrying one or no copy of this haplotype [additive model, p values between 0.0012 (adjusted for gender, gestational age, IGF-I, and birth weight for gestational age classified as ESGA, SGA, AGA, or LGA) and 0.0062 (adjusted for gender, gestational age, sum of IGF-I and IGF-II, and birth weight for gestational age classified as ESGA, SGA, AGA, or LGA)]. The haplotype CT was associated with a decrease in IGFBP-3 concentrations for individuals carrying two copies of the haplotype CT when compared to those carrying one or no copy of this haplotype [additive model, p values between 0.0347 (adjusted for gender, gestational age, IGF-I, and birth weight for gestational age classified as ESGA, SGA, AGA, or LGA) and 0.086 (adjusted for gender, gestational age, sum of IGF-I and IGF-II, and birth weight for gestational age classified as ESGA, SGA, AGA, or LGA)]. The haplo-

type CC was associated with a decrease in IGFBP-3 concentrations, however, not significantly.

We observed no significant associations between the *IGFBP3* promoter polymorphisms and the levels of IGF-I and IGF-II and no association between the *INS VNTR* polymorphism and the *IGF1* promoter polymorphism with cord plasma levels of IGF-I, IGF-II, or IGFBP-3.

Discussion

In this study, we investigated the influence of polymorphisms in the promoter regions of insulin, *IGF1*, and *IGFBP3* on cord plasma levels of IGFs and IGFBP-3 in 677 neonates. We observed strong associations of polymorphisms within the promoter of the *IGFBP3* gene and IGFBP-3 concentrations but no associations between the other polymorphisms and the measured cord plasma levels. Limitations of the study are that we included a total of 677 neonates which limits the power to detect smaller effects. Furthermore, the study is not strictly population based. We do not have any hint for but can neither definitely exclude a selection bias. As a technical restriction, it seems noteworthy that we used cord blood cells without prior cell sorting for genetic analyses as has been done previously [31, 32]. Although cord blood contains a mixture of different cell types, the pivotal cell population is of fetal origin.

IGFBP3 Polymorphisms

The influence of *IGFBP3* polymorphisms on circulating IGFBP-3 concentrations has been studied in several populations. For the -202 *IGFBP3* A/C polymorphism in the *IGFBP3* gene, a clear trend of decreasing IGFBP-3 concentration per copy of the C allele has consistently been reported in adult [5–10] and pediatric [11, 12] populations. Cord plasma levels of hormones and hormone-like substances, such as the IGFs and IGFBPs, reflect their intrauterine synthesis. In utero, however, IGFBP-3 synthesis underlies considerably different regulatory mechanisms as compared to postnatal life.

Despite these pronounced ‘environmental’ influences, the association between the two *IGFBP3* polymorphisms with IGFBP-3 concentration remained significant in all models even if we corrected for multiple hypotheses testing. To our knowledge, this is the first study demonstrating an association between these *IGFBP3* polymorphisms and IGFBP-3 concentration in cord plasma, which underscores the pronounced and stable genetic regulation of IGFBP-3 levels by this gene over life span.

In addition, we identified, for the first time, a significant association between the -185 *IGFBP3* polymorphism and cord plasma concentrations of IGFBP-3. This finding is in line with in vitro findings showing a significant haplotype-dependent transcriptional activity of both promoter polymorphisms [4]. Individuals carrying two copies of the most common haplotype AC in our study had the highest mean cord plasma IGFBP-3 levels. There was a difference of 8.4–12.2% in mean IGFBP-3 cord blood levels for the -185 C/T *IGFBP3* polymorphism as well as for the -202 A/C *IGFBP3* polymorphism between individuals homozygous for the rare allele compared to wild type carriers. These differences correspond to ten ‘centiles’, e.g. 10th to 20th percentile, in reference values for IGFBP-3 in newborns [36]. The influence of these two polymorphisms on IGFBP-3 levels is of interest not only with respect to growth regulation. In epidemiological studies, IGFBP-3 levels have been associated with cancer risk [5, 37, 38], and in vitro studies suggest a role for IGFBP-3 in the development of insulin resistance [39, 40].

Polymorphisms in the Insulin Gene and IGF1 Gene

The two polymorphisms investigated in the insulin gene and in the *IGF1* gene both had no effect on cord plasma concentrations of IGFs and IGFBP-3. Experimental data in term placenta had indicated that the *INS VNTR* polymorphism affects the expression of the *IGF2* gene [21] with class III alleles being associated with lower IGF-II transcription. This in vitro finding contrasts with findings from Ong et al. [41] who investigated cord blood levels of 353 neonates and reported slightly higher IGF-II levels for homozygote carriers of the class III alleles. In our markedly larger population of newborns, however, we observed no association between this polymorphism and IGF-II cord plasma concentration. In 1998, Dunger et al. [13] reported higher birth weights in class III homozygotes. This finding has not been confirmed in other populations [16, 17, 34, 42]. Absent associations between the *INS VNTR* polymorphism and birth weight are in line with absent associations between this polymorphism and IGF-II cord blood levels as demonstrated in our study. However, intrauterine growth is affected by many factors; thus, the results from genetic association studies can be very variable. Similarly, several mechanisms underlying fetal programming of adult disease such as programming of the cortisol axis [43] or of angiotensin receptors [44] have been suggested. Furthermore, postnatal growth and weight gain after intrauterine growth restriction have been shown to be in-

volved in developing metabolic syndrome associated with reduced fetal growth [45–47]. This could explain the discrepancy to prior publications.

The absence of the 192 bp allele of the *IGF1* promoter polymorphism has been linked to low birth weight [14], which was, however, not confirmed by other studies [15, 34]. Whether this polymorphism influences IGF-I levels in cord plasma has not been studied yet. In adults, conflicting results have been reported with respect to the influence of the 192 bp allele on circulating IGF-I concentrations [7, 15, 48, 49]. A recent study in 160 Japanese neonates did not find an association with IGF-I levels, which were, however, not measured in cord plasma but at day 5 after birth [50]. This finding is in line with our results. The Japanese group, however, identified an association between IGF-I concentrations and a 196-bp allele, which had a very low frequency (<1%) in our population.

Taken together, our data do not show associations between the *IGF1* promoter polymorphism or the investigated polymorphism in the insulin gene and IGF cord plasma levels. Our data thus do not support the hypothesis that the investigated polymorphisms influence IGF synthesis in utero and thereby affect birth weight. Genetic variants other than those investigated in our study might underlie the association between low birth weight and adult disease such as variants at loci near the gene encoding for cyclin L 1 (*CCNLI*) and the leucine, glutamate and lysine rich 1 (*LEKRI*) locus, in the *CDKALI* locus and in the genes encoding for adenylyl cyclase 5 (*ADCY5*) or for IGFBP2, which have all been described recently [44–46].

Conclusions

This is the first study showing a clear association between the *IGFBP3* promoter polymorphisms and IGFBP-3 cord plasma concentration. The study thus provides a first hint that also in utero, there is a stable influence of these polymorphisms on IGFBP-3 synthesis. Furthermore, our data do not provide evidence that the investigated polymorphisms in the insulin and in the *IGF1* gene influence IGF cord plasma levels or affect birth weight through IGF levels in utero.

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Disclosure Statement

The authors of this study have no conflict of interest and no financial interest that could be perceived as prejudicing the impartiality of the research reported.

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