Associations of *IGF-1* gene variants and milk protein intake with IGF-I concentrations in infants at age 6 months — Results from a randomized clinical trial

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Objective: The interplay of genetic and nutritional regulation of the insulin-like growth factor-I axis in children is unclear. Therefore, potential gene–nutrient effects on serum levels of the IGF-I axis in a formula feeding trial were studied.

Design: European multicenter randomized clinical trial of 1090 term, formula-fed infants assigned to receive cow’s milk-based infant and follow-on formulæ with lower (LP: 1.25 and 1.6 g/100 mL) or higher (HP: 2.05 and 3.2 g/100 mL) protein contents for the first 12 months of life; a comparison group of 588 breastfed infants (BF) was included. Eight single nucleotide polymorphisms (SNPs) of the *IGF-1*-(rs6214, rs1520220, rs978458, rs7136446, rs10735380, rs2195239, rs35767, and rs35766) and two of the IGFBP-3-(rs1496405, rs65670) gene were analyzed. Serum levels of total and free IGF-I, IGFBP-3 and the molar ratio IGF-1:IGFBP-3 at age 6 months were regressed on determined SNPs and feeding groups in 501 infants.

Results: *IGF-1-SNPs* rs1520220, rs978458, and rs2195239 significantly increased total-IGF-I and molar-ratio *IGF-I*:IGFBP-3 by ~1.3 ng/mL and ~1.3 per allele, respectively; compared to LP infants concentration and molar-ratio were increased in HP by ~1.3 ng/mL and ~1.3 and decreased in BF infants by ~0.6 ng/mL and ~0.6, respectively. IGFBP-3 was only affected by the BF group with ~450 ng/mL lower levels than the LP group. No gene-feeding-group interaction was detected for any SNP, even without correction for multiple testing.

Conclusions: Variants of the *IGF-1* gene play an important role in regulating serum levels of the IGF-I axis but there is no gene-protein-interaction. The predominant nutritional regulation of *IGF-I* level and IGFBP-3 gives further evidence that higher protein intake contributes to metabolic programming of growth.

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1. Introduction

Insulin-like growth factor-I (*IGF-I*) — a polypeptide hormone with structural and functional homology with proinsulin — is essential for mediating the metabolic, endocrine and anabolic effects of growth hormone (GH) on pre- and postnatal growth [1,2].

*IGF-I* derives mostly from the liver, but is also produced by other tissues [2]. The majority (~90%) of serum *IGF-I* is bound to IGF binding protein 3 (IGFBP-3) — one of six high affinity, soluble carrier binding proteins with the function to carry *IGF-I* in circulation, direct *IGF-I* to specific cell types, bind it to tissue specific receptors (e.g. *IGF-1R*) and...
modulate growth-promoting actions; e.g. by protein synthesis, affecting
cells size and cell number [1,3,4]. The ratio of IGF-I to IGFBP-3 is thought to
be a crude indicator of bioavailability of unbound free IGF-I [5].
Levels of serum IGF-I are not only stimulated by GH secretion but are
also influenced by genetic variations [2] and nutritional components
[6,7].
In adulthood, several studies investigated the genetic regulation of
blood levels of the IGF-I axis by the IGF-I and the IGFBP-3 genes
[8–15]. A recent genome-wide association study and a study tagging a
broad range of single nucleotide polymorphisms (SNP) have identified
novel loci related to circulating IGF-1 and IGFBP-3 in adults [10,12].
However, studies on genetic regulation of the IGF-I axis in children are
rare [16–18].
In addition, several studies reported reference values of IGF-I levels
in both healthy and growth hormone deficient children [19–22] includ-
ing reports on the nutritional regulation of IGF-I levels in children [6,7].
Recent research suggests that protein intake in early infancy may be a
main contributor for nutritional regulation of the IGF-I axis in infancy
and later childhood [5,23,24]. In fact, our group has recently shown in
a randomized clinical trial (European Childhood Obesity Project,
CHOP) that IGF-I and IGFBP-3 levels in 6 month old infants are explicitly
regulated by the amount (high vs. low) and not the type of protein
content in formula milk. The higher intake of branched-chain
-aminocids by feeding the higher protein formula resulted in higher
IGF-I but not IGFBP-3 levels [24].
However, despite this accumulated body of knowledge, the inter-
play of genetic and nutritional regulation of the IGF-I axis in children
is still not fully understood.
The aim of this study is therefore to investigate potential gene-
nutrient effects on serum levels of the IGF-I axis in this protein
formula feeding trial.

2. Subjects and methods

2.1. Study design

Details on the CHOP study design and procedures of this trial have
been published previously [24,25]. Briefly, the European Childhood
Obesity Trial Study is a double-blind, randomized, multicenter, ongoing,
prospective nutritional intervention trial (RCT) in infants conducted in
Germany, Belgium, Italy, Poland and Spain. Over the first year of life in-
fants of the two randomized groups were fed with an infant formula
and a follow-on formula (from age 5 months onwards) based on cow
milk with lower (LP) or higher protein (HP) content. Protein content in
the formulae (manufactured by Bledina, Steenvoorde, France, and
provided free of charge to families) was 1.25 and 1.6 g protein per
100 mL in the LP and 2.05 and 3.2 g/100 mL in the HP formula. All for-
mulae complied with the 1991 EU Directive on Infant and Follow-on
Formulae [26]. Protein contents of LP and HP formulae were chosen to
represent approximately the minimum and maximum of the EU Direc-
tive accepted ranges. Despite differences in protein content respective
of infant and follow-on formulae, the same energy density as, the differ-
ence in protein content was compensated by a corresponding ad-
justment of the fat content (see Table 1). An observational group of
exclusively breastfed infants (BF) was also included in this RCT as
randomized clinical trial (European Childhood Obesity Project,
CHOP) that IGF-I and IGFBP-3 levels in 6 month old infants are explicitly
regulated by the amount (high vs. low) and not the type of protein
content in formula milk. The higher intake of branched-chain
-aminocids by feeding the higher protein formula resulted in higher
IGF-I but not IGFBP-3 levels [24].
However, despite this accumulated body of knowledge, the inter-
play of genetic and nutritional regulation of the IGF-I axis in children
is still not fully understood.
The aim of this study is therefore to investigate potential gene-
nutrient effects on serum levels of the IGF-I axis in this protein
formula feeding trial.

2.2. Study population

Study population, randomization, allocation and follow-up of the
study participants for the first two years in the intervention and obser-
vation group have been reported in previous publications [24,25]. In
brief: between 1st October 2002 and 31st July 2004 1678 apparently
healthy, singleton, term infants were recruited in the study centers in
Munich and Nuremberg (Germany), Liege and Brussels (Belgium),
Milan (Italy), Warsaw (Poland) and Reus and Tarragona (Spain). Infants
of mothers with a hormonal or metabolic disease were not included
into the study. Eligible infants were enrolled during the first 8 weeks
of life (median age 14 days). The criteria of compliance to assigned
study group were exclusive formula-feeding at the end of the 8th
week of life for the protein formula groups or breastfeeding since
birth for the breastfeeding group, respectively. Non-compliance
resulted in exclusion from further study participation. At recruitment
breastfeeding was actively promoted and supported by the staff of all
study centers.
Final analysis population for the present study (n = 501) are all
those children of the originally enrolled infants (n = 1678) who
were still participating in the RCT at age 6 months (n = 1200) and
who also had valid data on both, measured serum levels of total
IGF-I, free IGF-I or IGFBP-3 (n = 764) and information on genetic
variants of the IGF-I or IGFBP-3 genes (n = 771) measured in buccal
cells.

2.3. Blood sampling

At the infant’s age of 6 months, a venous blood sample was drawn to
determine parameters of the IGF-I axis, except in the Italian study
centers where blood sampling at the age of 6 months was not ap-
proved by the local ethical committee. Parents were advised not to
feed their infant for at least 2 hours before the blood draw. Serum
samples were stored at −70 °C and transported on dry ice to one
central laboratory (The Children’s Memorial Health Institute, War-
saw, Poland).

2.4. DNA extraction and genotyping

DNA was extracted at the Research Unit of Molecular Epidemiology,
Helmholtz Zentrum München from dried buccal cell material spread on
FTA filter cards (Whatman GmbH, Dassel, Germany). Five 3 mm spots
containing buccal cell material were punched out of the cards and the
QiAmp DNA Micro Kit (Qiagen GmbH, Hilden, Germany) according to
the “Isolation of Genomic DNA from Dried Blood Spots” protocol
(without carrier RNA) was used for extraction. Genomic DNA was

Table 1
Composition of study formulae compared to human milk per 100 mL.

<table>
<thead>
<tr>
<th></th>
<th>Infant formula</th>
<th>Follow-on formula</th>
<th>Human milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low protein</td>
<td>High protein</td>
<td></td>
</tr>
<tr>
<td>Whey-Protein</td>
<td>20.80</td>
<td>20.80</td>
<td>60.20</td>
</tr>
<tr>
<td>Non-protein</td>
<td>2.05</td>
<td>2.05</td>
<td>1.2 ± 0.15</td>
</tr>
<tr>
<td>Lipids (g)</td>
<td>3.9</td>
<td>3.5</td>
<td>3.8 ± 0.96</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>6.7</td>
<td>7.5</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>69.9</td>
<td>69.8</td>
<td>74 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>60.7 ± 6.7</td>
<td>72.7</td>
<td>74 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>69.7 ± 6.7</td>
<td>72.5</td>
<td>70.7 ± 9.2</td>
</tr>
</tbody>
</table>

For further details on composition of study formulae see Table 1 of Socha et al. [24].

* Values according to Nommensen et al. [41]; values are means ± sd.
Fig. 1. Flow-chart of study population.
Genotyping assays were designed using the Assay Design 3.1 software and genotyping was performed by a MALDI-TOF MS based procedure using iPLEX™ Gold chemistry (Sequenom, San Diego, CA, USA). Only single nucleotide polymorphisms (SNPs) with a minor allele frequency > 10% were selected for genotyping. For the laboratory parameters (IGF-I, IGFBP-3), tagging SNPs (r² > 0.8) was based on genotype data from the HapMap database and SNPs that have previously been associated with the level of the respective parameter in literature, were selected [8,11,14,15,18].

2.5. Determination of parameters of the IGF-axis

Total IGF-I, free IGF-I, and IGFBP-3 were measured from serum samples using immunoradiometric assay kits (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) at the central laboratory of the Memorial Child Health Institute (Warsaw, Poland). For determination of IGF-I the IRMA kit (DSL-2800) was used. The sensitivity of the assay was 2.06 ng/mL. Intra- and inter-assay coefficients of variation (CV) were: 7.5 and 8.4%, respectively. No cross-reactivity of the anti-IGF antibody used in the assay with IGF-II, insulin, pro-insulin and growth hormone was declared by the manufacturer. For determination of IGFBP-3 the IRMA kit (DSL-6600) was used. The sensitivity of the assay was 0.5 ng/mL. Intra- and inter-assay coefficients of variation (CV) were: 3.8 and 5.9%, respectively. There was no cross-reactivity of the anti-IGFBP-3 antibody used with rhIGFBP-1, rhIGFBP-2, rhIGFBP-4-5 and 6, rhIGF-1 and II.

2.6. Outcome definition

Serum concentrations of total IGF-I, free IGF-I, and IGFBP-3 in nanogram per milliliter (ng/mL) and the molar ratio of total IGF-I/IGFBP-3 were used as outcome variables in the standard regression models. The molar ratio has been suggested by others [5] to reflect the bioavailable portion of IGF-I.

The molar ratio of IGF-I/IGFBP-3 was calculated after applying the conversion formulae reported in Hophet et al. [32]:

\[
1 \text{ng/mL IGF-I} - 0.133 \text{nM IGF-I} \quad \text{and}\n\]

\[
1 \text{ng/mL IGFBP-3} - 0.033 \text{nM IGFBP-3} \quad \text{and}\n\]

Standards and controls were calibrated to recombinant, non-glycosylated human IGFBP-3 (MW = 28.75 kDa).

2.7. Statistical analysis

Standard linear regression was applied to assess both the influence of genetic variants of the IGF-I and IGFBP-3 gene and the potential effect of the randomized LP and HP formula groups and the breastfed children (BF) on the outcomes serum levels of total IGF-I, free IGF-I, IGFBP-3 and on the molar ratio of IGF-I/IGFBP-3, respectively.

All models were adjusted for indicator (1/0) coded variables gender (male vs. female), any maternal smoking during pregnancy (yes vs. no), parental education (high vs. low and medium vs. low) and for continuously coded infant’s weight at age 6 months (in kg) to account for potential gender-specific differences in IGF-I levels [33], potential effects of these covariates on the nutritional regulation of IGF-I levels or found differences between formula-fed and breastfed feeding groups. Each SNP entered separately the regression equation as a continuous variable with homozygous major allele carriers coded as 0, heterozygous carriers coded as 1 and homozygous minor allele carriers coded as 2. The indicator coded variables for the HP formula group and for the BF group were entered in the regression model to directly test the influence of the HP vs. the LP formula and the BF group vs. the LP formula group in addition to the potential genetic influence on the respective IGF-I axis outcome.

To account for multiple testing in these regression models conservative Bonferroni corrections were applied; i.e. P-values in Table 5 were multiplied by the number of analyzed outcomes times the number of different predictors (i.e. 4 × 9 = 36).

Allele frequencies and Fisher’s exact test for Hardy-Weinberg Equilibrium (HWE) for all available genetic data (n = 771) and the analysis population (n = 501) were calculated by the software JLIN version 1.6 [34].

Deviations from normal distributions of the four continuous outcome variables were evaluated by Kolmogorov-Smirnov-tests and by Q-Q- and stem-and-leaf-plots. Severely right-skewed outcomes (total IGF-I, free IGF-I and the molar ratio of IGF-I/IGFBP-3), were transformed by base 10 logarithm.

To assess the relative contribution of genetic and nutritional factors regarding the regulation of the IGF-I axis related outcome, regression models were built, which hierarchically partition the explained variance (R²) according to the contributions of covariates (gender, maternal smoking in pregnancy, parental education and infant’s weight at age 6 months), genetic variants and nutrition type (HP, LP, and BF). That is, first separate models were estimated for covariates, the six IGF related SNPs and nutrition type. Then hierarchical models were estimated and the explained variance of the genetic, respective nutritional variance contribution in addition to the already accounted variance of several covariates (gender, maternal smoking, parental education and infant’s weight at age 6 months) for the respective outcome was evaluated. Finally the additional variance of the nutrition type was assessed after covariables and all six genetic variants were already in the model.

Formal sensitivity analyses were conducted to ensure representativeness of the analyzed data of 501 mother-child pairs with those 1177 not in the analyzed sample and those 764 with IGF measurement but no genotyping. The effects of feeding type, being participant of the analyzed data set (yes/no) and the interaction of these two variables were tested on all covariables of Table 2 and all IGF parameters in Table 4 by linear and logistic regression as appropriate. Significant interaction terms indicate relevant differences in the group of children analyzed and those originally participating.

All analyses except evaluation of properties of SNPs were conducted using the statistical software SAS, version 9.2 [35].

3. Results

The characteristics of the analysis population stratified by group allocation are listed in Table 2.

Birth weight and length for boys and girls were not significantly different between the randomized groups of LP and HP formulae (P-values from two-sided t-test P < 0.82, P < 0.40 and P < 0.94, P < 0.14, respectively); neither birth weight of mothers between the two protein formula groups differed (P-value from two-sided t-test P < 0.69). Only paternal birth weight was significantly higher in the HP than in the LP formula group (P-value from two-sided t-test P < 0.02). No differences were detected for percentage of any smoking during pregnancy, regarding low, medium and high parental education and regarding the four study centers between the LP and HP formulae groups (P-values from two-sided Wald-test from logistic regression P > 0.18 and P < 0.14, P < 0.83, P < 0.26 and P < 0.59, P < 0.84, P < 0.48, P < 0.77, respectively). Hence, with the exception of the higher paternal birth weight in the HP group there is no indication that randomization could have been dissolved by restriction to this subsample.

Comparing the randomized formula groups with the breastfed group some significant differences were found (see Table 2). Breastfeeding was significantly more common in the Belgian and less so in the Spanish study centers, respectively. Mothers of breastfed children smoked less during pregnancy and parents of breastfed children were significantly
higher educated. To adjust for potential confounding we adjusted later multivariate analysis by these variables.

Characteristics of all genotyped SNPs for the IGF-1 and IGFBP-3 genes are displayed in Table 3.

Genotyping success rate was excellent except for the IGF-1 gene related SNP rs6214. Although all genotypes had reasonable large allele frequencies, Fisher's exact test for Hardy–Weinberg-Equilibrium (HWE-test) revealed that rs6214 and three other SNPs (rs35767, rs35766) violated this equilibrium assumption in this analysis population ($n = 501$). This was also true if HWE was tested for the total number of children with genetic data ($n = 771$). Only total IGF-I and the molar ratio IGF-I/IGFBP-3 were significantly affected by any of the analyzed polymorphisms. Carriers of one minor allele in rs1520220, rs978458 or rs2195239 increased the log 10 transformed total IGF-I by about 0.11 (i.e. ~1.29 ng/mL) and for the log 10 transformed molar ratio IGF-I/IGFBP-3 by about 0.22 (i.e. ~1.66 ng/mL) if the infant was a carrier of two minor alleles (see Table 5).

Table 3
Characteristics of variants of the IGF-1 and IGFBP-3 genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>dbSNPBuild 132</th>
<th>Position, bp</th>
<th>Location</th>
<th>Alleles(major/minor)</th>
<th>Number (%) of subjects withgenotype1</th>
<th>Genotyping success</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6214</td>
<td>101,317,699</td>
<td>3′ UTR</td>
<td>Intronic</td>
<td>C/A</td>
<td>157</td>
<td>157</td>
<td>185</td>
</tr>
<tr>
<td>rs1520220</td>
<td>101,320,652</td>
<td>Intronic</td>
<td>C/G</td>
<td>320</td>
<td>148</td>
<td>20</td>
<td>97.9</td>
</tr>
<tr>
<td>rs978458</td>
<td>101,326,369</td>
<td>Intronic</td>
<td>C/A</td>
<td>247</td>
<td>187</td>
<td>41</td>
<td>94.9</td>
</tr>
<tr>
<td>rs7136446</td>
<td>101,362,645</td>
<td>Intronic</td>
<td>T/C</td>
<td>161</td>
<td>219</td>
<td>102</td>
<td>96.9</td>
</tr>
<tr>
<td>rs10735380</td>
<td>101,368,366</td>
<td>Intronic</td>
<td>A/G</td>
<td>239</td>
<td>202</td>
<td>33</td>
<td>95.4</td>
</tr>
<tr>
<td>rs2195239</td>
<td>101,380,832</td>
<td>Intronic</td>
<td>C/T</td>
<td>283</td>
<td>161</td>
<td>33</td>
<td>96.3</td>
</tr>
<tr>
<td>rs35766</td>
<td>101,399,699</td>
<td>5′ near gene</td>
<td>C/T</td>
<td>327</td>
<td>126</td>
<td>24</td>
<td>95.8</td>
</tr>
<tr>
<td>rs35766</td>
<td>101,404,603</td>
<td>5′ near gene</td>
<td>A/G</td>
<td>333</td>
<td>131</td>
<td>27</td>
<td>97.7</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1486495</td>
<td>45,903,786</td>
<td>Intergenic</td>
<td>T/C</td>
<td>332</td>
<td>111</td>
<td>21</td>
<td>94.6</td>
</tr>
<tr>
<td>rs6670</td>
<td>45,918,779</td>
<td>3′ UTR</td>
<td>A/T</td>
<td>290</td>
<td>161</td>
<td>22</td>
<td>95.7</td>
</tr>
</tbody>
</table>
two minor alleles. SNP rs10735380 showed also a significant effect regarding the molar ratio (0.09 per minor allele; untransformed 1.23).

For SNP rs6670 related to the IGFBP-3 gene no significant and substantial effect could be detected for any outcome after the feeding type was included in the gender and covariate adjusted model.

### 3.2. Nutritional effects

Infants fed with the HP formula generally had increased levels for total IGF-I, free IGF-I and for the molar ratio IGF-I/IGFBP-3 in comparison to the LP formula group (see Table 5). These effects were generally substantially higher than the genetic effects if compared to carriers of one minor allele only, but slightly lower in comparison to genetic effects of carriers of two minor alleles.

Regarding levels of IGFBP-3, no significant or substantial difference could be detected between the formula groups.

Breastfed infants show generally for all four outcomes (total IGF-I, free IGF-I, IGFBP-3, molar ratio IGF-I/IGFBP-3) substantially and except for outcome free IGF-I also significantly lower values than the LP formula group. Likewise the comparison of the BF group to the HP formula group revealed substantially and also significantly lower values for all four outcomes.

The effect of feeding type (HP, LP, and BF) on all parameters of the IGF-I axis was not dependent on the SNP polymorphism, as no

### Table 4
Mean and 95% confidence interval of IGF-I-Total, IGF-I-Free and IGFBP-3 serum levels stratified by feeding group.

<table>
<thead>
<tr>
<th>Low Protein Formula</th>
<th>High Protein Formula</th>
<th>Breastfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 189</td>
<td>n = 187</td>
<td>n = 125</td>
</tr>
<tr>
<td><strong>IGF-I Total</strong></td>
<td><strong>IGF-I Free</strong></td>
<td><strong>IGF-BP3</strong></td>
</tr>
<tr>
<td>(ng/mL)</td>
<td>(ng/mL)</td>
<td>(ng/mL)</td>
</tr>
<tr>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td>45.17</td>
<td>0.60</td>
<td>2968.06</td>
</tr>
<tr>
<td>40.06–50.28</td>
<td>0.52–0.67</td>
<td>2866.33–3069.79</td>
</tr>
<tr>
<td><strong>Molar ratio IGF-I / IGFBP-3</strong></td>
<td><strong>log10 (IGF-I Total)</strong></td>
<td><strong>log10 (IGF-I Free)</strong></td>
</tr>
<tr>
<td>–</td>
<td>(ng/mL)</td>
<td>(ng/mL)</td>
</tr>
<tr>
<td>0.06</td>
<td>1.51</td>
<td>–0.35</td>
</tr>
<tr>
<td>0.05–0.07</td>
<td>1.45–1.57</td>
<td>−0.4 to −0.3</td>
</tr>
<tr>
<td>0.08</td>
<td>1.66</td>
<td>−0.23</td>
</tr>
<tr>
<td>0.07–0.09</td>
<td>1.61–1.72</td>
<td>−0.29 to −0.18</td>
</tr>
<tr>
<td>0.04</td>
<td>1.22</td>
<td>−0.46</td>
</tr>
<tr>
<td>0.03–0.05</td>
<td>1.14–1.3</td>
<td>−0.51 to −0.41</td>
</tr>
</tbody>
</table>

### Table 5
Regression of serum IGF-I and IGFBP-3 levels on genetic variants of the IGF-I and IGFBP-3 genes and feeding groups.

<table>
<thead>
<tr>
<th>log10 (IGF-I-Total)</th>
<th>log10 (IGF-I-Free)</th>
<th>IGFBP-3</th>
<th>log10 (IGF-I / IGFBP-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>S.E.(R)</td>
<td>P-Value</td>
<td></td>
</tr>
<tr>
<td>0.68</td>
<td>0.17</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>1.22</td>
<td>0.14</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>1112.60</td>
<td>293.77</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>0.13</td>
<td>0.04</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>0.04</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>436.07</td>
<td>89.29</td>
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</table>

\[a\] P-values are conservatively corrected by multiplying each uncorrected P-value by 36 (i.e. 4 outcomes × 9 predictors) to account for multiple testing.

\[b\] Note each regression model is adjusted for infant’s weight at 6 months and indicator coded variables maternal smoking in pregnancy, high and low parental educational level (reference low education of both parents) for the reasons described in the method section.
interaction between feeding type and available SNPs could be detected (data not shown).

### 3.3. Relative importance of genetic and nutritional effects

In Table 6 we summarized the variation (R²) on parameters of the IGF-I axis explained by covariates (gender, maternal smoking in pregnancy, parental education, and infant’s weight at age 6 months), feeding type and genetic variants. All six SNPs taken together explain 3% to 5% of the variance of the respective outcome in addition to variation already explained by the covariates. We found that the explained variation (R²) of the feeding type in addition to the already explained variation of the genetic variants (all six SNPs together) and the covariates were about four-fold for total IGF-I (10.9%/2.7%), 1.5-fold (3.8%/2.5%) for free IGF-I and 2.6-fold (10.5%/4.0%) for the molar ratio IGF-I/IGFBP-3.

### 3.4. Sensitivity analyses

Formal sensitivity analysis revealed no significant differences between the analyzed and non-analyzed population for the formula-fed groups. However, for breastfed children there were significant differences for maternal smoking during pregnancy and higher parental education [data not shown]. This indicates that in the analysis population comprising only 501 mother-child pairs there were a somewhat lower percentage of breast feeding mothers, who smoked during pregnancy and a higher proportion of breastfeeding mothers with higher education than in the non-analyzed sample.

### 4. Discussion

This randomized, clinical trial (RCT) showed that serum levels of the IGF-I axis are not regulated by IGF-1 or IGFBP-3 gene–protein intake-interaction in 6 months old infants. Rather, genotype and protein intake affected serum levels of the IGF-I axis independently from each other. In particular, only total IGF-I and the molar-ratio IGF-I/IGFBP-3 are genetically influenced by IGF-1 gene related single nucleotide polymorphisms (SNPs) rs1520220, rs978458, and rs2195239, when protein intake by feeding type and covariate adjustment for infant’s weight at age 6 months, maternal smoking during pregnancy and parental education were taken into account. HP contents formula milk enhanced independently from genetic effects total and free IGF-I serum levels as well as the molar ratio IGF-I/IGFBP-3, but not the level of IGFBP-3; whereas BF children generally had lower values in all studied IGF-I axis related parameters. Serum levels of the IGFBP-3 were only affected by BF (substantially lower compared to formula-fed children), but not by any of the analyzed polymorphisms related to the IGFBP-3 or IGF-1 gene.

### 4.1. Effects of genetic variants of the IGF-1 and IGFBP-3 genes

Not all previously shown effects of the genetic variants on the IGF-I axis could be reproduced. Regarding genetic regulation of the IGF-I axis by the IGF-1 and the IGFBP-3 genes most knowledge is based on studies in adults from cancer or cardiovascular research [8–15] and on IGF-1-knock-out mice studies [2]. However, studies on IGF-1 or IGFBP-3 gene related regulation of IGF-I or IGFBP-3 serum levels in children are rare [16–18]. During the first year of life nutritional factors have more pronounced effects on the IGF-I axis than later in life [6,7]. Furthermore, infants with high IGF-I serum levels tend to have lower IGF-I levels later in life [36,37]. Thus, factors regulating IGF-I levels in infancy and adulthood differ which might well explain, why we did not detect any effect for several of the analyzed SNPs.

Two previous studies have explored the effect of IGF-I and IGFBP-3 SNPs on serum levels of the IGF-I axis in children. Johnston et al. found a significant association between childhood serum IGF-I values and the genotype of the intron 2 CT dinucleotide repeat marker (IGF-1/PCRI) in a group of 114 Swedish pre-pubertal children born small for gestational age (SGA), but not in normal birth weight controls [16]. Children of the SGA group, who were homozygous carriers of the major allele of the IGF-1/PCRI genotype had significantly lower serum IGF-I levels than those with other genotypes. Moreover, no other genetic marker (3′ microsatellite marker D12S3189, promoter CA repeat IGF-1/1737.738, single nucleotide polymorphism IGF-1/17148C, single nucleotide polymorphism IGF-1/A + 1771C) was associated with IGF-I standardized levels.

A second study in 292 pre-pubertal Dutch children being SGA showed significantly higher IGFBP-3 levels standardized to reference values (IGFBP-3-3DS), if they were carriers of the homozygous minor allele of the −202 A/C IGFBP-3 promoter SNP [17]. Though not directly comparable because of analysis of different genetic variants in SGA children, we also found significantly lower levels for total IGF-I in homozygous major allele carriers for three of the five SNPs tested. However, in contrast to the analysis of van der Kaay et al. [17], we did not find a significant nor substantial effect for the SNP rs6670, located in the 3′ untranslated region of IGFBP-3.

Nevertheless, we explained about 3–5% of the total variance of IGF-I related parameters by our 6 gene variants. This is in line with Vella et al. who analyzed the association of 7 tagging SNPs related to the IGF-1 gene (rs35140968, rs6214, rs3730220, rs6219, rs2946831, rs3730204, and rs12579108) and IGF-I concentrations in about 300 newborns in a British birth cohort in the UK [18]. In their study about 6% of variation in IGF-1 concentrations were explained by these 7 SNPs in a multilocus regression analysis similar to ours presented in Table 6. However, the examined SNPs were different from those in our study, with the exception of rs6214 which had a low genotyping success rate in our study and was not analyzed.

**Table 6**

<table>
<thead>
<tr>
<th>Covariates</th>
<th>log10 (IGF-I-Total)</th>
<th>log10 (IGF-I-Free)</th>
<th>log10 (IGF-I / IGFBP-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R² in %</td>
<td>R² in %</td>
<td>R² in %</td>
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<tr>
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<td>15.0</td>
</tr>
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<td>Nutritional only</td>
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<tr>
<td>Genetic contribution in addition to covariates</td>
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<td>3.6</td>
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<tr>
<td>Nutritional contribution in addition to covariates &amp; genetic</td>
<td>10.9</td>
<td>3.8</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Genetic variables comprise SNPs rs1520220, rs978458, rs7136446, rs10735380, rs2195239 and rs6670. Nutritional variables are higher protein contents infant formula (HP) group and breastfeeding (BF) group indicator coded variables (reference is lower protein formula group (LP)).

R² — contribution due to e.g. "nutrition in addition to covariates & genetics" means the additional explained variance of HP and BF after covariates and genetic variables have already been accounted for in a hierarchically built regression model (see Subjects and methods section).

* Covariates are indicator coded variables gender (male = 1 vs. female = 0), any maternal smoking during pregnancy (yes = 1 vs. no = 0), two parental education variables (high = 1 vs. low = 0 and medium = 1 vs. low = 1) and the continuously coded variable infants weight at 6 months in kilogram.
Although genetic effects were small in comparison to the nutritional influence, 3.8%, 3.6%, and 5.0% of the variance in total IGF-I, free IGF-I, and IGF-I/IGFBP-3 levels, respectively, could be genetically explained. This explanatory power is rather high for genetic association studies. However, the exact mechanisms how these genetic variants influence the IGF-I axis are unclear. Similarly, the effect of protein intake on circulating IGF-I has been reported previously by us [24], but the exact molecular mechanisms and involved pathways remain speculative.

From our association results between IGF-I variants and IGF-I levels, it is difficult to deduce molecular mechanisms or even the causal variant, which is responsible for the observed association. According to the Broad Institute’s SNP annotation and Proxy Search tool (http://www.broadinstitute.org/mpg/snap/ldsearch.php), which searches for correlations between polymorphisms in the 1000 Genomes pilot 1 data set, several SNPs in the IGF-I gene region are highly correlated with our associated SNPs ($r^2 > 0.8$); and could be responsible for the observed associations. Some of these are located in potential regulatory regions (including conserved transcription factor binding sites, DNase hypersensitive sites, and histone mark regions) according to the UCSC genome browser (http://genome.ucsc.edu/index.html?org=Human&db=hg18&hgsid=215421565). However, since these possible functionalities are mainly based on bioinformatic predictions and high-throughput methods, causality can only be inferred from detailed molecular characterization of individual SNP effects by functional studies. Deal et al. investigated the functional effects of IGFBP-3 promoter polymorphisms and reported an allele-dependent effect on promoter activity of the −202 polymorphism (rs2854744) in an in vitro assay, suggesting that this SNP may be causally involved in IGFBP-3 expression and circulating protein levels. However, the association of rs6670 seen in our study may only be explained in part by this promoter SNP; there is little correlation between the −202 promoter polymorphism and our investigated SNP ($r^2 = 0.042$, 1000 Genomes pilot 1 dataset). We, therefore, speculate that additional functional variants might exist [38]. We hypothesized that the effect of protein intake on the IGF-I axis might be more pronounced by polymorphisms in SNPs that have already been shown to regulate the IGF-I axis. Our literature research did not result in publications analyzing simultaneously genetic and nutritional effects on serum levels of the IGF-I axis. Therefore, we cannot compare our result of no gene-protein interaction with previous publications.

### 4.2. Effects of higher protein content formula milk

Previous studies, including one study of our research group, have already shown that higher protein contents in formula milk increased levels of total and free IGF-I in infancy, while breastfeeding resulted in lower (total and free) IGF-I levels for breastfed infants [5,24,37,39]. In the present study we could show that these differences are even when genetic regulation of IGF-I levels and several nutrition and otherwise related covariates (infant’s weight at age 6 months, parental educational level, maternal smoking in pregnancy) are taken into account, emphasizing the nutritional aspect of IGF-I regulation. Moreover, our results are in line with the recently published elevated levels of IGFBP-3 and molar ratio IGF-I/IGFBP-3 for formula-fed infants of age 9 months and substantially lower values in breastfed infants [5].

### 4.3. Relevance of these results

We found that the explained variation ($R^2$) in total and free IGF-I, and in the molar ratio IGF-I/IGFBP-3 was remarkably higher for feeding type than for genetic variants: Feeding type explained 4-fold more variance than genetic variants for total IGF-I, about 1.5-fold more for free IGF-I and about 2.6-fold more for the total IGF-I/IGFBP-3 ratio, even when all six SNPs and several nutrition or otherwise IGF related covariates were predictors in the model (see Table 6). This predominant nutritional regulation of total and free IGF-I and the molar ratio IGF-I/IGFBP-3 gives further evidence that HP intake contributes to metabolic-programming of the IGF-I axis and potentially later growth [25,36,37].

### 4.4. Strength

To the best of our knowledge this is the first study which investigates both nutritional and genetic regulation of levels of parameters of the IGF-I axis and the effects of a potential gene–protein intake interaction in infants in one study. Most studies only investigated either nutritional or genetic effects in humans [26,67] and even fewer studied either issue in children or infants [5,16–18,24,37–41]. Moreover, the present study is a large and well powered double-blind RCT enabling the detection of causal relationships, whereas most studies are observational only or investigate only few children. Furthermore, only the total protein content, but not the amino acid composition differed among the HP and LP content formula. Thus, in contrast to some previous studies the whey-casein ratio is identical and it can be excluded that type of protein instead of the amount of protein content affects IGF-I levels.

### 4.5. Limitations

Whereas five SNPs related to the IGF-I gene were available and met the assumptions for a single-SNP regression analysis, only one polymorphism related to the IGFBP-3 gene could be used due to violations of the HWE-test. Thus it cannot completely be ruled out that other variants of the IGFBP-3 gene may regulate IGFBP-3 levels.

Except for a higher paternal weight at birth in the HP formula group comparison of study characteristics showed that there were no indications that the analyzed subset of the study population could have adversely affected randomization. However, we consider this unlikely as birth weights of both girls and boys did neither substantially nor significantly differ.

Although, sensitivity analyses to check for potential sample selection bias between the analyzed 501 mother–child pairs and the not included 1177 mother child pairs described in the results section did reveal some differences, we do not consider these differences to have substantially compromised representativeness of our reduced analysis sample. Moreover, the main results of Table 5 were adjusted for several nutrition- or otherwise IGF-related covariates (gender, maternal smoking during pregnancy, parental educational level and weight at 6 months) to account for potential differences.

Therefore, we consider the selected analysis population as sufficiently representative of the whole cohort. Most important, this selection did not dissolve the randomization of the effects of the high vs. low protein formula groups. Nevertheless, we cannot completely rule out that some sample selection bias could be present.

In conclusion: Serum IGF-I levels are not regulated by gene-protein intake-interaction in 6 months old infants. Only total IGF-I and the molar-ratio IGF-I/IGFBP-3 are genetically influenced when protein intake is taken into account. The variation of free IGF-I and IGFBP-3 is predominantly modulated by nutrition rather than genetic variation, which provides further support for the relevance of early protein supply on metabolic-programming of the IGF-I axis and growth.

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Conflict of interest statement

The participating company had no decisive role in the conduct and analysis of the study. None of the authors reports a conflict of interest, following the guidelines of the International Committee of Medical Journal Editors.

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