FOOD-CT-2005-007036

EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity: 3.5.4
Title of activity: Human studies and genomics

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5, 5 Years

Organisation Name of Lead Contractor for this report: Helmholtz

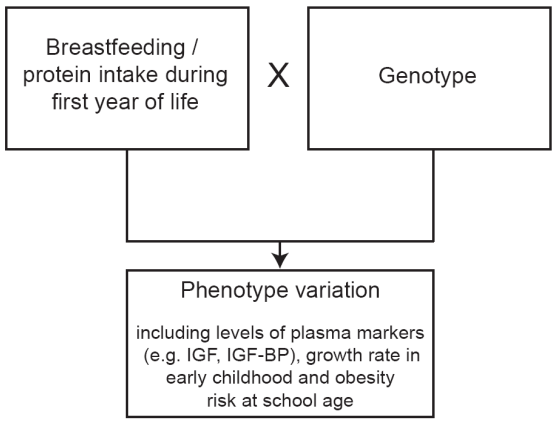
Background

The CHOP (CHildhood Obesity Project) study investigates the relationship between protein intake in early life and later obesity risk in a randomized double blind controlled intervention trial where formula fed children were randomly allocated to get formula milk with higher (HP) or lower protein (LP) content during the first year of life; a reference group of breastfed children (BF) was also observed. At the age of 12 and 24 months, infants fed the higher protein diet had a significantly higher weight-for-length z-score (according to WHO reference data) than the lower protein and breastfed group (1). Additionally, the higher protein group exhibited enhanced levels of plasma IGF-1, lower concentrations of IGF-BP2, and higher urinary excretion of insulin C-peptide (2, 3). These results support the hypothesis that higher protein intake is associated with increased insulin secretion and stimulation of IGF-axis-parameters. The study has been followed up in the course of the EARNEST project to analyze if these relationships remain until school age and also if there is a relation with overweight/obesity in school-aged children since high weight gain during the second year of life is associated with later risk for obesity (4).

Main objective

The main objective of the activity 3.5.4 during the complete project period was to determine whether the genetic make-up (single nucleotide polymorphisms, SNPs) affects circulating levels of markers such as IGF and IGF-BP2 or phenotypes such as BMI or obesity in the CHOP children and, especially, whether the already observed effects of protein intake on these markers are modulated by genetic variation.

Figure 1: Hypothesis to be tested in activity 3.5.4.



Experimental design

DNA extraction

Buccal cell samples were collected from all children, whose parents declared their consent for genetic analysis, and were spread onto FTA filter cards for long-term storage at room temperature. For DNA extraction from the filter cards, we used the QiaAmp DNA Micro Kit (Qiagen) according to the "Isolation of Genomic DNA from Dried Blood Spots" protocol after some optimization steps to obtain DNA of sufficient quality and quantity for genotyping. In total, 802 DNA samples were extracted and available for genotyping (158 from Germany, 180 from Poland, 224 from Spain, 143 from Italy and 97 from Belgium). The amount of DNA obtained from the filter cards was sufficient for genotyping a maximum number of 50-60 SNPs.

Candidate gene and SNP selection

For genotyping, SNPs were selected that have been previously shown to be associated with:
a. laboratory parameters measured in the CHOP study;
b. obesity and/or BMI;

c. insulin resistance or diabetes;
d. lipid pathways.
To ensure a sufficient number of homozygous minor allele carriers for proper statistical analysis SNPs have to provide a minor allele frequency of at least 10%. For the laboratory parameters (INS-IGF2, IGF1, IGF-BP3, IGF-BP2, ADIPOQ), tagging SNPs (r^2>0.8) based on genotype data from the HapMap database and SNPs that have previously been associated with levels of the respective parameters in literature, were selected. For the BMI, diabetes, and lipid genes, the selection was based on literature research focusing on phenotypes relevant for the CHOP study (e.g. BMI) in genome-wide association or candidate gene studies. A detailed list of selected genes and SNPs is shown in Table 1.

Genotyping
Genotyping assays were designed using the Assay Design 3.1 (Sequenom) software. In some cases, assay design failed for single SNPs and for these SNPs, tagging SNPs (r^2>0.8) were selected instead. Three assays were designed for a total number of 49 SNPs (including two gender-specific SNPs for quality control). The two gender-specific SNPs and 43 of the 47 candidate SNPs were genotyped successfully. For data analyses five of these 43 SNPs were excluded because cluster separation between genotype groups was not optimal resulting in a mean genotyping success rate of 84% among those SNPs in contrast to the mean genotyping success rate of 95% for all others. Duplicate and gender discordance rates were in the normal range. Eleven samples showed discordant genders between DNA analysis and database entry and were excluded from analyses as well as 20 duplicated samples and two samples which could not be allocated to the children. Finally, there is genotype data for 769 children with 38 measured SNPs. Genotyping success rates, allele frequencies and p-values of the test on Hardy-Weinberg-Equilibrium are summarized in Table1.

Table 1: Candidate genes and SNPs that were genotyped in the CHOP study

	Gene	SNP	Call-rate	Comment	Alleles	Major / Minor allele frequency	Missings (%)	p-value HWE-test
Insulin resistance / Diabetes	CDKAL1	rs10946398	95,7		A>C	0,67 / 0,33	4,2	0,57
	CDKN2B	rs10811661	93,6		T>C	0,83 / 0,17	6,4	0,44
	HHEX	rs10882102	94,3		G>C	0,61 / 0,39	5,6	0,82
	IGF2BP2	rs4402960	94,3		G>T	0,68 / 0,32	5,6	0,035
	KCNJ13	rs2074314	97,9		A>G	0,63 / 0,37	2,0	0,49
	MTNR1B	rs10830963	89,5		C>G	0,73 / 0,27	10,0	0,19
	PPARG	rs1801282	96,3		C>G	0,89 / 0,11	3,6	0,85
	SLC30A8	rs3802177	79,5	bad cluster separation	C>T	0,74 / 0,26	20,0	0,00084
	TCF7L2	rs4132670	95,9		C>T	0,67 / 0,33	4,0	0,11
Laboratory parameters	ADIPOQ	rs2241766	96,5		T>G	0,88 / 0,12	3,2	0,056
	ADIPOQ	rs17366568	93,3		G>A	0,88 / 0,12	6,6	0,39
	ADIPOQ	rs1501299	74	genotyping failed				
	IGF1	rs6214	87,8	bad cluster separation	G>A	0,59 / 0,41	12,0	0,00035
	IGF1	rs35766	97,7		A>G	0,81 / 0,19	2,2	0,0032
	IGF1	rs35767	95,8		C>T	0,82 / 0,18	4,1	0,027
	IGF1	rs1520220	97,9		C>G	0,81 / 0,19	2,0	0,2
	IGF1	rs2195239	96,3		G>C	0,78 / 0,22	3,7	0,3
	IGF1	rs7136446	96,9		T>C	0,57 / 0,43	3,1	0,31
	IGF1	rs10735380	95,4		A>G	0,73 / 0,27	4,5	0,36
	IGF1	rs978458	94,9		G>A	0,73 / 0,27	4,9	0,46
	IGF1	rs1019731	76	genotyping failed				
	IGFBP2	rs9341134	97		A>T	0,91 / 0,085	3,0	0,0022

	IGFBP2	rs1525608	90,9		C>T	0,57 / 0,43	9,1	0,0024
	IGFBP2	rs3770473	98,6		A>C	0,7 / 0,3	1,2	0,053
	IGFBP2	rs7603372	96,9		T>C	0,64 / 0,36	3,0	0,31
	IGFBP2	rs2270360	82,5	bad cluster separation	T>G	0,68 / 0,32	18,0	0,93
	IGFBP3	rs1496495	94,6		T>C	0,84 / 0,16	5,4	0,0073
	IGFBP3	rs6670	95,7		A>T	0,79 / 0,21	4,4	1
	INS-IGF2	rs3842748	98,3		C>G	0,77 / 0,23	1,6	0,61
	INS-IGF2	rs689	97,9		A>T	0,72 / 0,28	2,0	1
Lipid genes	FADS3	rs174550	96		T>C	0,7 / 0,3	3,7	0,27
Obesity / BMI	BCDIN3D, FAIM2	rs11169176	95,9		G>A	0,54 / 0,46	4,0	0,082
	FTO	rs12149832	92,2		G>A	0,61 / 0,39	7,7	0,1
	FTO	rs6499640	92,7		A>G	0,61 / 0,39	7,2	0,64
	MAF	rs1424233	89,4		G>A	0,51 / 0,49	10,0	0,18
	MC4R	rs502933	96,5		C>A	0,67 / 0,33	3,4	0,12
	MC4R	rs12967135	1,7	genotyping failed				
	NEGR1	rs2568958	97,2		A>G	0,66 / 0,34	2,7	0,13
	NPC1	rs1805081	91,6		A>G	0,62 / 0,38	8,4	0,05
	PRL	rs4712652	91,6		A>G	0,58 / 0,42	8,4	0,82
	SDCCAG8	rs10926984	96,3		T>G	0,87 / 0,13	3,7	0,64
	SDCCAG9	rs12145833	86,4	bad cluster separation	T>G	0,85 / 0,15	14,0	0,23
	SFRS10, ETV5, DGKG	rs7635103	79,1	genotyping failed				
	SH2B1	rs7498665	96,5		A>G	0,64 / 0,36	3,4	0,35
	TMEM18	rs12714415	84,3	bad cluster separation	T>C	0,91 / 0,092	16,0	2,80E-26
	TNKS	rs17150703	98,1		G>A	0,92 / 0,083	1,7	0,1
	TNKS	rs13278851	92,8		G>A	0,92 / 0,081	7,2	0,13

Statistical analysis

Effects of genotypes were assessed according to the additive model. Correction for multiple testing was performed using the Holm method. Genotype frequencies were tested to be equally distributed between feeding groups and countries to be aware of confounding effects.

Results

1. IGF-axis-parameters measured at the age of 6 months

Fourteen of the 38 SNPs showed an association with at least one of the IGF-axis-parameters according to literature. Six of them were excluded for a Hardy-Weinberg-Disequilibrium. IGF1-total (-free) levels were significantly associated with 5 (2) SNPs (rs978458, rs10735380 [only total IGF1], rs2195239 [only total IGF1], rs1520220 [only total IGF1] and rs7136446). Additional models with country and gender were fitted but adjustment did not change the results for IGF1-total and -free.

For the IGF binding proteins no significant association could be detected with correction for multiple testing. Only the SNP rs3770473 showed a tendency to influence IGF-BP2 levels (p-value of linear regression without correction for multiple testing: 0.023; adjusted for country and gender: 0.013) and IGF-BP3 levels tended to increase with the minor allele frequency of the SNP rs1496495 (crude p-value: 0.016, adjusted: 0.031).

Higher protein intake increased IGF1-total and -free levels, reduced IGF-BP2, and did not affect IGF-BP3 levels (2). To analyse if genotypes modulate the effect of protein on these parameters of the IGF-axis, first formula type and then the interaction between formula type and genotype was included in the model; only formula fed children were used for analysis.

There are two questions to be answered:

Does the effect of higher protein on IGF-axis-parameters persist if genetic variation is embedded in the model?
The effect of a higher protein intake on IGF1-total and IGF-BP2 was not affected by any SNP. However, the effect of protein intake on IGF1-free levels was no longer significant after adjustment for genotypes. Since there have been no significant associations between genotypes and levels of IGF-BP2 or -BP3, adjustment for genotype did not have any effect on the association with formula type.

Do the different genetic variants modify the effect of protein intake on IGF-axis-parameters?
For IGF1-total, IGF-BP2 and -BP3, we did not detect modulating effects by genotypes.
For IGF1-free some modulating effect of genotypes could be seen. Although statistical significance was not achieved, the effect of formula type on IGF1-free levels was generally higher in the homozygote minor allele group ([p-value for interaction between genotype and formula group] rs7136446 [0.04], rs10735380 [0.03], rs1496495 [0.01], rs3770473 [0.03]).

2. Birth weight and BMI

Most of the SNPs selected because of their known associations with BMI or obesity are also known to be associated with birth weight. These findings were not reproducible in the CHOP cohort.

Since the study initially was designed to see a difference in anthropometry at the age of 2 years, associations between genotypes and BMI at 24 months of age were analysed and no significant effects could be found. Also for modifying effects of protein on associations between genotype and BMI was tested but no interrelationships could be justified.
Genetic effects on growth development are shown to increase over time and reach statistical significance only for children older than 4 years in longitudinal models (4).
Therefore, it is not surprising that modelling growth paths for the different SNPs did not reach statistical significance. Nevertheless, some tendency for an effect has been seen in some SNPs (rs12149832, rs4712652, rs11169176, rs10926984, rs17150703, rs4132670, rs10946398, and rs10882102).

Conclusions

The data on the genetic make-up enables us to further analyse metabolic structures underlying the "Early protein hypothesis". The effect of protein intake seems to be influenced by genotype. However, the programming effects of higher protein intake during the first year of life on anthropometry in relation to genotypes need to be studied more in detail, especially for the later periods of the CHOP study. Since parameters to assess diabetes risk have not been available yet, further analysis regarding this topic will be carried out in 2011.

Outlook

Following these children up until teenage will provide the possibility to assess the effectiveness of a lower protein diet on the risk of obesity. Adjustment for genetic variation which showed to play a more important role with increasing age will be of help in further assessment of anthropometry, metabolic structures and diabetes risk parameters.

1. Koletzko B et al. Lower protein in infant formula is associated with lower weight up to age 2 y: a rondomized clinical trial. AJCN 2009; 89: 1836-45
2. Socha P et al. Milk protein intake and the metabolic-endocrine response in infancy – a randomized clinical trial
3. Toschke AM et al. Identifying children at high risk for overweight at school entry by weight gain during the first 2 years. Arch Pediatr Adolesc Med 2004; 158: 449-52

4. Rzehak P et al. Associations between BMI and the FTO gene are age dependent: results from the GINI and LISA birth cohort studies up to age 6 years. Obes Facts 2010; 3: 173-180