

**SERUM LEPTIN AND LEPTIN RECEPTORS IN HEALTHY PREPUBERTAL CHILDREN: RELATIONS TO INSULIN RESISTANCE AND LIPID PARAMETERS, BODY MASS INDEX (BMI), TUMOR NECROSIS FACTOR  $\alpha$  (TNF $\alpha$ ), HEART FATTY ACID BINDING PROTEIN (hFABP), AND IgG ANTICARDIOLIPIN (ACL-IgG)**

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In a group of randomly selected 29 healthy prepubertal children (16 boys, mean age  $9.56 \pm 0.7$  years, 13 girls, mean age  $9.96 \pm 0.9$  years) fasting serum leptin and leptin receptor concentrations were measured by ELISA and compared with insulin parameters (homeostatic model of assessment insulin resistance = HOMA IR, insulin, intact proinsulin, C-peptide) and some metabolic parameters and factors that contribute to insulin resistance: triacylglycerols, high density lipoprotein cholesterol (HDL cholesterol), low density lipoprotein cholesterol, body mass index, tumor necrosis factor, heart fatty acid binding protein, and IgG fraction of anticardiolipin. Statistical analysis was performed using SAS/STAT software and included analysis of normality, analysis of variance, Spearman's correlations, linear and multiple regression analysis with insulin parameters as dependent variables. The subgroups of boys and girls did not differ significantly in any of parameters studied. Serum concentrations of insulin, intact proinsulin, HOMA IR, C-peptide and triacylglycerols appeared to be primarily influenced by serum leptin concentration. Serum leptin concentrations were tightly correlated with body mass indexes and negatively correlated with leptin receptor concentrations, probably as a manifestation of down regulation. The role of other factors studied appeared to be complementary or less significant (hFABP, ACL IgG), or absent (TNF $\alpha$ ). We concluded that in healthy prepubertal children of both genders serum leptin concentration contributes to insulin resistance and to insulin resistance-related metabolic changes.

## INTRODUCTION

Leptin is a protein coded by the *ob* gene and released by adipose tissue into the circulation. Leptin depresses appetite and influences energy homeostasis, thus leading to a decrease in body weight. The concentration of leptin in plasma is closely correlated with the amount of body fat and body mass index<sup>1</sup>. Leptin molecules bind to a hypothalamic receptor, which triggers changes of gene expression (downregulation of neuropeptide Y and upregulation of corticotropin-releasing factor) and increases sympathetic nervous activity. Leptin also acts on peripheral tissues, regulating leptin gene expression, fatty acid synthesis and triacylglycerol oxidation. The opposite effects, i.e. increased food intake, conservation of energy and storage of body fat, are associated with a decline of circulating leptin or with a leptin resistance. In general, obese human subjects have high leptin levels and their obesity is associated with insensitivity to leptin<sup>2</sup>.

Prepubertal girls and boys show a similar pattern of serum leptin increase in parallel to increased body weight and BMI until the age of nearly 10 years<sup>3</sup>. Girls have

slightly higher absolute leptin concentrations than boys even in this prepubertal period, which can reflect the gender differences of body composition and fat distribution<sup>4</sup>. In boys serum leptin decreases at the moment of testosterone increase, in girls the pubertal increase of serum leptin values is followed by rising estrogen levels. Peripheral administration of leptin accelerated onset of puberty in female mice<sup>5</sup>.

Insulin plays a distinct role in the regulation of leptin synthesis<sup>6, 7, 8</sup>. A chronic increase in the plasma insulin level results in increased leptin production in man<sup>9</sup>. In turn, leptin stimulates insulin secretion in pancreatic B-cells<sup>10</sup>. In adipose tissue leptin diminishes insulin effect on glucose transport, glycogen synthesis, and lipid synthesis, while increasing lipolysis<sup>11</sup>. In liver, leptin enhances glycogene storage and gluconeogenesis while suppressing glycogen breakdown and diminishing flux through glucose-6-phosphatase. In some respect, its hepatic actions contribute to insulin resistance<sup>12</sup>. In muscles, leptin stimulates glucose uptake and glycogen synthesis independent on insulin action<sup>13</sup>.

Serum leptin levels were higher in diabetic than in healthy children<sup>14</sup>. These differences were not attribu-

table to age, adiposity or stage of pubertal development, and were probably conditioned by the metabolic perturbation intrinsic to the diabetic state, or by the chronic hyperinsulinemia.

Some authors at the 59<sup>th</sup> Annual Scientific Sessions of the American Diabetic Association, held in June 1999, focused on changing demographics of type 2 diabetes and the metabolic syndrome. There is an epidemic of this syndrome in young children, associated with a marked increase of insulin resistance, obesity and hypertension in these children<sup>15, 16, 17, 18</sup>. In order to understand the role of leptin and to reveal possible early symptoms of these alterations we measured serum leptin and leptin receptors in prepubertal children and correlated them with insulin parameters, body mass indexes and some other factors that are connected with insulin resistance and its metabolic consequences: tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), heart muscle fatty acid binding protein (hFABP) and IgG antidiolipin (ACL IgG). In this paper, results from healthy children are presented.

## SUBJECTS AND METHODS

*Subjects.* The study was carried out on 29 randomly selected healthy children, 16 boys with a mean age of  $9.56 \pm 0.7$  years, and 13 girls with a mean age of  $9.96 \pm 0.9$  years. None of the children had clinical or laboratory signs of a disease, they were taking no drugs. Body mass indexes (BMI) defined as weight in kilograms divided by the square of height in meters ( $\text{kg}/\text{m}^2$ ) were calculated. A written informed consent was obtained from the parents of the children. Blood samples were obtained in the morning after overnight fasting by venopuncture. After clotting the serum was separated and stored at  $-20^\circ\text{C}$  until assay.

*Leptin and leptin receptor assays.* Serum leptin levels were measured by ELISA commercial test kits, which is an enzyme-linked immunosorbent assay based on the sandwich principle (Human Leptin ELISA, BioVendor Laboratory Medicine, Inc, Czech Republic). The limit of sensitivity was 0.2 ng/ml, the intra-assay CV was 6.1 % at the level of 7.5 ng/ml, and the inter-assay CV was 8.5 % at the level of 4.8 ng/ml. Serum leptin receptor concentrations were measured by a newly developed set (Human Leptin Receptor ELISA, BioVendor), which is a double sandwich method using a pair of mice monoclonal antibodies. The limit of sensitivity was 1.8 ng/ml, the intra-assay CV was 4.6% at the level of 30.4 ng/ml and the inter-assay CV was 7.8 % at the level of 32.1ng/ml. In both methods, TMB was used as a substrate, recombinant leptin receptor as a standard and quality controls were human serum based. In a previous study, performed with this set, the serum leptin receptor concentrations in the adult population ranged within 40–50 ng/ml<sup>21</sup>.

*Other methods.* Several other hormones and peptides were estimated by routine double sandwich ELISA sets: insulin (CLIA-IMMULITE, DPC), intact proinsulin

(DAKO Intact Proinsulin), C-peptid (IMMULITE, DPC), Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ , IMMULITE, DPC), antidiolipin IgG antibodies (ACL-IgG, IMMCO Diagnostics), and heart fatty acid binding proteins (H-FABP, Hbt HUMAN H-FABP, HyCult Biotechnology b.v.). At the same time, the concentrations of glucose, cholesterol, triacylglycerols, HDL-cholesterol, LDL-cholesterol, Apoprotein B and uric acid were measured on ILAB-600 biochemical analyser using BioVendor sets. All samples were processed and examined according to principles of correct laboratory practice.

The homeostatic indexes of insulin resistance (HOMA IR) and homeostatic indexes of B-cells secretory activity (HOMA  $\beta$  cell) were calculated as follows<sup>19, 20</sup>:

$$\text{HOMA IR} = \text{Fasting insulin } (\mu\text{U}/\text{ml}) * \text{Fasting glucose } (\text{mmol}/\text{l}) / 22.5;$$

$$\text{HOMA } \beta \text{ cell} = 20 * \text{Fasting insulin } (\mu\text{U}/\text{ml}) / \text{Fasting glucose } (\text{mmol}/\text{l}) - 3.5.$$

*Statistics.* Statistical analyses were performed using the Version 6 SAS/STAT software (SAS Institute Inc., Cary, NC, USA). In testing the normality of distribution the Shapiro-Wilk's test was used instead of Kolmogorov's test, because the sample size for data was less than 80. Most of the data obtained were not normally distributed. Spearman's rank-order correlation was therefore used for correlation analysis. Linear regression and multiple regression analyses were performed using the insulin-resistance parameter (HOMA IR) and insulin-secretion parameters (fasting serum insulin, and C-peptide) as dependent variables, and various other hormonal and metabolic factors (leptin, leptin receptors, TNF $\alpha$ , hFABP, ACL IgG, BMI and lipid parameters) as independent variables. The so called 2 Step-down regression model was used to select dominant independent variables. First, a multiple regression was performed using a group of explanatory variables. This group should not include too many variables, because the relationships become too complex to interpret. Variables were then dropped, one at a time (At each stage, the variable chosen for exclusion is that which makes the least contribution to the explained variation). The process continued until remaining variables were significant. The coefficient of determination  $R^2$  can be viewed as a percentage explaining the total variance. A great drop in simultaneously calculated  $R^2$  enabled us to select the most dominant determinant of the dependent variable.

## RESULTS

Table 1 shows the characteristics of all subjects under study. Normal distribution, proved by Shapiro-Wilk's study, could not be excluded only in a minority of data sets, i.e. in uric acid, glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and leptin receptor serum concentrations. In the remaining characteristics, including all insulin parameters, TAG, leptin, TNF $\alpha$ , ACL IgG and hFABP, the normal distribution was excluded.

We compared the data between the groups of boys and girls using the *t* test in normal distributed data sets, and Kolmogorov-Smirnov's test in comparing data when at least in one of the data sets compared the normal distribution was excluded. The differences between all parameters studied were insignificant, including serum leptin concentration (The usually described higher concentration of leptin in girls even in the prepubertal age was probably abolished by the presence of one boy with somewhat higher BMI and leptin concentration among our probands). Because we wanted to study the common mechanisms of insulin resistance and associated metabolic changes, we decided to analyze the results of both genders together.

**Table 1.** Characteristics of subjects under study; normal distribution of data was proven by Shapiro-Wilks test

	n	Mean	SD	Normal distribution
Age	29	9.62 (years)	0.9029	No
BMI	29	17.08	3.2742	No
Uric acid	29	256.07 (µmol/l)	49.5587	Yes
Glucose	29	5.19 (mmol/l)	0.5288	Yes
Cholesterol	29	4.52 (mmol/l)	0.6274	Yes
Triacylglycerol	29	0.96 (mmol/l)	0.4469	No
HDL-cholest.	29	1.59 (mmol/l)	0.3400	Yes
LDL-cholest.	29	2.49 (mmol/l)	0.5668	Yes
ApoB	29	0.84 (g/l)	0.1420	Yes
Insulin	29	10.65 (mIU/l)	4.9460	No
HOMA IR	29	2.4971	1.3410	No
HOMA βcell	28	124.5929	55.6482	No
Proinsulin int.	29	3.573(pmol/l)	2.5384	No
C-peptide	29	0.67(nmol/l)	0.2655	No
Leptin	29	6.00 (ng/ml)	8.2767	No
Leptin Recept.	29	30.61 (ng/ml)	6.7639	Yes
TNF α	19	14.32 (pg/ml)	5.6070	No
ACL IgG	29	3.24 (IU/ml)	2.8035	No
hFABP	29	196.32 (pg/ml)	200.6251	No

**BMI** = body mass index, **ApoB** = apoprotein B. **HOMA IR** = homeostasis model of insulinoreistance. **HOMA β cell** = homeostasis model of β-cells insulin secretion. **Leptin Recept.** = serum leptin receptors. **TNFα** = tumor necrosis factor α, **ACL IgG** = IgG anticardiolipin. **hFABP** = heart muscle fatty acid binding protein.

Table 2 shows Spearman's correlations between insulin characteristics and metabolic variables. The positive correlations between BMI on the one side and leptin, C-peptide and HOMA IR on the other side are highly statistically significant. So are also the correlations between TAG and C-peptide, and between leptin on the one hand, and HOMA IR, C-peptide and insulin on the other hand. A highly significant negative correlation exists between TAG and HDL-cholesterol. Somewhat less significant positive correlations exist between TAG on the one side and HOMA IR, leptin and BMI on the other side, and between hFABP on the one side and HOMA IR and C-peptide on the other side. There exists a significant positive correlation between TNFα and hFABP. Significant negative correlations were found between HDL cholesterol and C-peptide, and between leptin receptors on the one side and BMI and leptin on the other side. The p values of correlation coefficients between leptin receptor and a group of insulin parameters (insulin, HOMA IR, proinsulin) give reason to suspect some tendency to a negative correlation between these parameters. Only the serum concentration

of proinsulin and of ACL- IgG did not correlate with any of the parameters studied. However, a tendency to negative correlation between ACL-IgG and C-peptide seemed to be present (*p* = 0.06).

**Table 2.** Spearman correlations between insulin parameters and metabolic variables

	BMI	TAG	HDL-cholest.	LDL-cholest.	Leptin	Leptin Recept.	TNFα	ACL-IgG	hFABP
Insulin	<i>r</i> <sub>s</sub> =0.54 <i>p</i> =0.002	<i>r</i> <sub>s</sub> =0.35 <i>p</i> =0.059	<i>r</i> <sub>s</sub> =-0.29 <i>p</i> =0.12	<i>r</i> <sub>s</sub> =-0.27 <i>p</i> =0.15	<i>r</i> <sub>s</sub> =0.47 <i>p</i> =0.009	<i>r</i> <sub>s</sub> =-0.32 <i>p</i> =0.083	<i>r</i> <sub>s</sub> =0.14 <i>p</i> =0.56	<i>r</i> <sub>s</sub> =-0.25 <i>p</i> =0.18	<i>r</i> <sub>s</sub> =0.44 <i>p</i> =0.15
Pro-Insulin	<i>r</i> <sub>s</sub> =0.04 <i>p</i> =0.82	<i>r</i> <sub>s</sub> =0.21 <i>p</i> =0.26	<i>r</i> <sub>s</sub> =-0.12 <i>p</i> =0.53	<i>r</i> <sub>s</sub> =-0.15 <i>p</i> =0.41	<i>r</i> <sub>s</sub> =-0.002 <i>p</i> =0.99	<i>r</i> <sub>s</sub> =-0.32 <i>p</i> =0.087	<i>r</i> <sub>s</sub> =-0.34 <i>p</i> =0.15	<i>r</i> <sub>s</sub> =-0.005 <i>p</i> =0.98	<i>r</i> <sub>s</sub> =-0.22 <i>p</i> =0.25
HOMA IR	<i>r</i> <sub>s</sub> =0.49 <i>p</i> =0.006	<i>r</i> <sub>s</sub> =0.39 <i>p</i> =0.036	<i>r</i> <sub>s</sub> =-0.28 <i>p</i> =0.14	<i>r</i> <sub>s</sub> =-0.18 <i>p</i> =0.36	<i>r</i> <sub>s</sub> =0.51 <i>p</i> =0.004	<i>r</i> <sub>s</sub> =-0.32 <i>p</i> =0.085	<i>r</i> <sub>s</sub> =0.10 <i>p</i> =0.67	<i>r</i> <sub>s</sub> =-0.005 <i>p</i> =0.09	<i>r</i> <sub>s</sub> =0.36 <i>p</i> =0.054
C-Peptide	<i>r</i> <sub>s</sub> =0.51 <i>p</i> =0.004	<i>r</i> <sub>s</sub> =0.60 <i>p</i> =0.005	<i>r</i> <sub>s</sub> =-0.43 <i>p</i> =0.018	<i>r</i> <sub>s</sub> =-0.02 <i>p</i> =0.92	<i>r</i> <sub>s</sub> =0.48 <i>p</i> =0.008	<i>r</i> <sub>s</sub> =-0.27 <i>p</i> =0.15	<i>r</i> <sub>s</sub> =-0.37 <i>p</i> =0.12	<i>r</i> <sub>s</sub> =-0.35 <i>p</i> =0.06	<i>r</i> <sub>s</sub> =0.39 <i>p</i> =0.033
Leptin	<i>r</i> <sub>s</sub> =0.79 <i>p</i> =0.0001	<i>r</i> <sub>s</sub> =0.40 <i>p</i> =0.031	<i>r</i> <sub>s</sub> =-0.21 <i>p</i> =0.26	<i>r</i> <sub>s</sub> =-0.26 <i>p</i> =0.18	-	<i>r</i> <sub>s</sub> =-0.44 <i>p</i> =0.015	<i>r</i> <sub>s</sub> =0.26 <i>p</i> =0.65	<i>r</i> <sub>s</sub> =0.08 <i>p</i> =0.65	<i>r</i> <sub>s</sub> =0.33 <i>p</i> =0.07
Leptin Recept.	<i>r</i> <sub>s</sub> =0.41 <i>p</i> =0.025	<i>r</i> <sub>s</sub> =0.04 <i>p</i> =0.81	<i>r</i> <sub>s</sub> =-0.30 <i>p</i> =0.11	<i>r</i> <sub>s</sub> =-0.31 <i>p</i> =0.093	-	-	<i>r</i> <sub>s</sub> =-0.10 <i>p</i> =0.42	<i>r</i> <sub>s</sub> =0.09 <i>p</i> =0.63	<i>r</i> <sub>s</sub> =-0.13 <i>p</i> =0.48
TNFα	<i>r</i> <sub>s</sub> =0.30 <i>p</i> =0.21	<i>r</i> <sub>s</sub> =0.35 <i>p</i> =0.14	<i>r</i> <sub>s</sub> =-0.36 <i>p</i> =0.12	<i>r</i> <sub>s</sub> =-0.44 <i>p</i> =0.055	-	-	<i>r</i> <sub>s</sub> =-0.80 <i>p</i> =0.75	<i>r</i> <sub>s</sub> =0.57 <i>p</i> =0.010	-
ACL IgG	<i>r</i> <sub>s</sub> =-0.05 <i>p</i> =0.76	<i>r</i> <sub>s</sub> =-0.28 <i>p</i> =0.14	<i>r</i> <sub>s</sub> =-0.05 <i>p</i> =0.79	<i>r</i> <sub>s</sub> =0.08 <i>p</i> =0.67	-	-	-	-	<i>r</i> <sub>s</sub> =-0.09 <i>p</i> =0.64
hFABP	<i>r</i> <sub>s</sub> =0.35 <i>p</i> =0.061	<i>r</i> <sub>s</sub> =-0.01 <i>p</i> =0.96	<i>r</i> <sub>s</sub> =-0.19 <i>p</i> =0.32	<i>r</i> <sub>s</sub> =-0.26 <i>p</i> =0.16	-	-	-	-	-
BMI	-	<i>r</i> <sub>s</sub> =0.40 <i>p</i> =0.029	<i>r</i> <sub>s</sub> =-0.25 <i>p</i> =0.18	<i>r</i> <sub>s</sub> =0.002 <i>p</i> =0.99	-	-	-	-	-
TAG	-	-	<i>r</i> <sub>s</sub> =-0.62 <i>p</i> =0.0003	<i>r</i> <sub>s</sub> =0.02 <i>p</i> =0.89	-	-	-	-	-
HDL-cholest.	-	-	-	<i>r</i> <sub>s</sub> =0.08 <i>p</i> =0.66	-	-	-	-	-

*r*<sub>s</sub> = Spearman rank correlation coefficient. Statistically significant values are denoted by thick numbers. Abbreviations are the same as in Table 1.

Table 3 shows the results of linear regression analysis between most metabolic factors, taken as independent variables, and HOMA-IR, proinsulin, and C-peptide, taken as dependent variables. In the presence of HOMA IR as a dependent variable only the BMI and leptin R<sup>2</sup> was sufficiently high, leptin being the dominant independent variable. In the presence of proinsulin as a dependent variable none of the independent variables was sufficiently high; leptin, leptin receptor and BMI were the dominant independent variables. In relation to C-peptide as a dependent variable, BMI, leptin and TAG seemed to play a certain role in explaining this variable.

We attempted to apply logistic multiple regression analysis to our results with insulin resistance-related parameters as dependent variables. In a model with HOMA IR, leptin exerts a statistically high significance in influencing this variable (see upper part of Table 4). By step-by-step omission of individual independent variables (and simultaneous evaluation of the drop of R<sup>2</sup>) we could conclude, that ACL IgG but not the remaining variables (TNFα, hFABP) could play some role in explaining the HOMA IR changes. Among metabolic variables (HDL cholesterol, TAG, LDL cholesterol, BMI), only body mass index related significantly to HOMA IR; omission of HDL-cholesterol, TAG, and LDL-cholesterol did not markedly decrease R<sup>2</sup> (see Table 4, bottom).

Table 5 shows some results of other models of multiple regression analysis. C-peptide was again significantly influenced by serum leptin concentration and by BMI. hFABP and TAG could play also a certain role while the influence of TNF $\alpha$ , ACL IgG, HDL-cholesterol and LDL was clearly insignificant. Serum concentration of proinsulin depended significantly on serum leptin concentration and ACL IgG (note a drop of R<sup>2</sup> in bottom part of Table 5), while hFABP and TNF $\alpha$  seem not to play a significant role.

Leptin receptors did not play a significant role in any model of multiple regression analysis when insulin resistance-related parameters were taken as dependent variables (data not shown).

**Table 3.** Linear regression between HOMA IR, proinsulin and C-peptide, taken as dependent variables, and various individual metabolic factors, taken as independent variables

		HDL	TAG	LDL	BMI	Leptin	Leptin receptor	TNF $\alpha$	hFABP	ACL IgG
HOMA IR	Int.	4.4507	1.7237	3.1429	-2.2983	1.7962	4.9585	2.9187	2.2226	2.8265
	Par.	-1.2284	0.8004	-0.2591	0.2807	0.1167	-0.0129	-0.0185	0.0014	-0.1017
	R <sup>2</sup>	0.0970	0.0711	0.0120	<b>0.4696</b>	<b>0.5190</b>	0.1645	0.0049	0.0438	0.0452
Pro-insulin	Int.	4.7769	2.9390	1.5605	-2.4362	2.5693	9.5253	5.9255	3.7029	4.0274
	Par.	0.7565	0.6570	0.8076	0.3518	0.1673	-0.0311	-0.1174	-0.0007	-0.1401
	R <sup>2</sup>	0.0103	0.0134	0.0325	<b>0.2059</b>	<b>0.2976</b>	<b>0.2684</b>	0.0521	0.0027	0.0239
C-peptide	Int.	1.2569	0.3273	0.6764	-0.1906	0.5451	1.0940	0.5127	0.5563	0.7227
	Par.	-0.3769	0.3418	-0.0075	0.0496	0.0187	-0.0023	0.0116	0.0005	-0.0201
	R <sup>2</sup>	0.2330	<b>0.3310</b>	0.0003	<b>0.3749</b>	<b>0.3414</b>	0.1319	0.0521	0.1521	0.0451

Int. = intercept. Par. = parameter. R<sup>2</sup> = coefficient of determination (proportion of explained variations). Dominant independent variables are denoted by thick numbers. Other abbreviations are the same as in Table 1.

**Table 4.** Multiple regression analysis of data when homeostatic index of insulin resistance HOMA IR was considered as dependent variable, and leptin, TNF $\alpha$ , hFABP, ACL Ig, BMI and lipid parameters as independent variables

		Intercept	Leptin	TNF $\alpha$	hFABP	ACL-IgG	R <sup>2</sup>
HOMA IR	Par.	3.1102	0.1207	-0.0370	0.0006	-0.2788	<b>0.6921</b>
	p	0.0007	<b>0.0002</b>	0.3754	0.6409	<b>0.0221</b>	
HOMA IR	Par.	3.1830	0.1233	-0.0334		-0.2933	<b>0.6871</b>
	p	0.0005	<b>0.0001</b>	0.4012		<b>0.0111</b>	
HOMA IR	Par.	2.2816	0.1270			-0.1689	<b>0.6397</b>
	p	0.0001	<b>0.0001</b>			<b>0.0066</b>	
HOMA IR	Par.	1.7962	0.1167				<b>0.5190</b>
	p	0.0001	0.0001				
HOMA IR	Par.	-0.5234	-0.5147	-0.3020	-0.2483	0.2780	<b>0.4904</b>
	p	0.8106	0.5037	0.6153	0.4843	<b>0.0003</b>	
HOMA IR	Par.	-1.0893	-0.2898		-0.2263	0.2699	<b>0.4849</b>
	p	0.5557	0.6372		0.5138	<b>0.0002</b>	
HOMA IR	Par.	-1.6836			-0.2439	0.2803	<b>0.4802</b>
	p	0.2112			0.4725	<b>0.0001</b>	
HOMA IR	Par.	-2.2983				0.2807	<b>0.4696</b>
	p	0.0292				<b>0.0001</b>	

p value evaluates probability, that the independent variable has no effect on the dependent variable. Significant values of p and R<sup>2</sup> are denoted by thick numbers. Other abbreviations are the same as in Table 1 and Table 3.

**Table 5.** Multiple regression analysis of selected data when C-peptide and proinsulin were taken as the dependent variables

		Intercept	Leptin	TNF $\alpha$	hFABP	ACL-IgG	R <sup>2</sup>
C-peptide	Par.	0.5044	0.0176	0.0070	0.0004	-0.0356	<b>0.5571</b>
	p	0.0088	<b>0.0062</b>	0.4605	0.2055	0.1747	
C-peptide	Par.	0.5457	0.0195		0.0004	-0.0247	<b>0.5228</b>
	p	0.0001	<b>0.0002</b>		0.0548	0.0831	
C-peptide	Par.	0.4604	0.0179		0.0005		<b>0.4605</b>
	p	0.0001	<b>0.0007</b>		<b>0.0241</b>		
C-peptide	Par.	0.5451	0.0187				<b>0.3414</b>
	p	0.0001	<b>0.0009</b>				
C-peptide	Par.	-0.0505	-0.0956	0.1967	0.0242	0.0357	<b>0.5182</b>
	p	0.9044	0.5187	0.0978	0.7219	<b>0.0094</b>	
C-peptide	Par.	0.0091	-0.0948	0.1917		0.0359	<b>0.5156</b>
	p	0.9808	0.5144	0.0975		<b>0.0076</b>	
C-peptide	Par.	-0.2020		0.2350		0.0370	<b>0.5071</b>
	p	0.3077		<b>0.0138</b>		<b>0.0052</b>	
C-peptide	Par.	-0.1906				0.0496	<b>0.3749</b>
	p	0.3821				<b>0.0004</b>	
Proinsulin	Par.	6.3380	0.1938	-0.1388	-0.0003	-0.4560	<b>0.4955</b>
	p	0.0052	<b>0.0053</b>	0.1869	0.9377	0.1113	
Proinsulin	Par.	6.3079	0.1927	-0.1403		-0.4500	<b>0.4953</b>
	p	0.0020	<b>0.0033</b>	0.1602		0.0906	
Proinsulin	Par.	3.2480	0.1816			-0.2362	<b>0.3635</b>
	p	0.0001	<b>0.0010</b>			0.1130	
Proinsulin	Par.	2.5693	0.1673				0.2976
	p	0.0001	<b>0.0022</b>				

Abbreviations are the same as in the previous Tables.

## DISCUSSION

In 1988, Reaven suggested that insulin resistance may underlie a number of diseases, including hypertriglyceridemia accompanied by lowering of HDL lipoprotein level, hypertension, impaired glucose tolerance leading to type 2 diabetes mellitus, and hyperinsulinemia<sup>22</sup>. In nondiabetic subjects the increased insulin concentrations reflect insulin resistance<sup>23</sup>. The metabolic consequences of hyperinsulinemia tend to cluster forming "syndrome X". Even in non-diabetic individuals, both insulin resistance and compensatory hyperinsulinemia are associated with increases in hepatic VLDL-triacylglycerol secretion and elevated plasma TAG concentration, followed by lowering of plasma HDL levels and by increased forming of smaller denser atherogenic LDL particles. These changes are the most common manifestation of insulin resistance and compensatory hyperinsulinemia, responsible for the increased risk of CHD in these individuals<sup>24, 25</sup>.

In the present study, we attempted to analyse the possible role of leptin and leptin receptors and several other factors in regulating insulin secretion and insulin resistance in healthy prepubertal children of both genders. Insulin secretion was judged by measuring serum insulin, proinsulin and C-peptide concentration. Insulin resistance was quantified on the basis of the homeostatic model of assessment (HOMA IR), which was proven as a method to assess insulin resistance from the fasting insulin and glucose concentrations<sup>20</sup> and which was correlated with hyperinsulinemic euglycemic clamp<sup>19</sup>. Reli-

able assessing of insulin secretion by the homeostatic model (HOMA  $\beta$ cell) required a second blood specimen obtained 30 min after the 75-g oral glucose load and was therefore not used in the correlations in accordance with our endeavour to achieve maximal simplicity and minimal invasivity of the whole procedure.

Serum leptin concentrations were positively correlated with insulin, C-peptide, HOMA IR, BMI, TAG and negatively correlated with leptin receptors (See Table 2). Increased leptin production that parallels the quantity of adipose tissue (see the very high correlation between BMI and leptin) is accompanied by a tendency to increased insulin resistance and its metabolic consequences, and to compensatory insulin production (high insulin and C-peptide). The inverse correlation between leptin and leptin receptors may be a consequence of down regulation.

Linear regression analyses between these variables showed also the prominent role of serum leptin concentration (and BMI) in explaining insulin resistance and secretory activity of  $\beta$  cells, while the role of serum TAG concentration and leptin receptors seems to be less important or secondary (see Table 3). The results of multiple regression analysis of our data must be considered very cautiously regarding the relatively low number of observations. Nevertheless, the important role of leptin and BMI in explaining HOMA IR, C-peptide and intact proinsulin is indubitable (See Tables 4 and 5). The role of ACL-IgG in influencing HOMA IR and intact proinsulin, and the role of hFABP and TAG in influencing C-peptide seems to be also significant.

These results support the concept of an important physiological role of leptin as an inhibitor of insulin secretion; a failure of this inhibitory function, accompanied by hyperleptinemia, may explain – at least in animal experiments with ob/ob and db/db mice – the development of hyperinsulinemia, insulin resistance, and the progression glucose intolerance<sup>26</sup>. In humans, hyperleptinemia is an early sign of juvenile obesity (it is not a chronic adaptation to the obese state) and is closely linked to subcutaneous fat mass<sup>27</sup>. Changes in leptin concentration in individuals with impaired glucose tolerance were best explained by changes in fat mass among both adult males and females<sup>28</sup>. However, results of studies dealing with interrelationships between plasma insulin and leptin are controversial. Some studies have found increases of leptinemia over 3–4 h of hyperinsulinemia and during insulin resistance-related metabolic changes<sup>29, 30, 31</sup> while others have found no relation of leptin concentration to hyperinsulinemia and its consequences<sup>32, 32</sup>. These variable findings could be explained by studying groups of probands of different age and gender, suffering with different metabolic alterations, and by application of various research methods. The individual differences in plasma leptin during hyperinsulinemia may be also related to the relative lipolytic or lipogenic state of certain subcutaneous adipose tissue depots. Mechanisms for regulation of leptin expression in this tissue may share some of the pathways that

regulate lipolysis<sup>34</sup>. In prepubertal healthy children the sex hormones and corticoids don't play a dominant role as in pubertal or adult subjects and physiological metabolic and regulatory pathways prevail. According to our results, the leptin expression in adipose tissue plays a dominant role in these mechanisms and is related to such important metabolic parameters as the degree insulin sensitivity (insulin resistance) and insulin secretion.

The cytokine TNF $\alpha$  is also expressed primarily in adipose tissue, where it is an important modulator of gene expression. Its expression increases with increasing adiposity in rodents and humans<sup>35, 36</sup> and correlates strongly with hyperinsulinemia<sup>37</sup>. TNF $\alpha$  has been reported to increase the circulating leptin concentration in humans<sup>38</sup>. TNF $\alpha$  inhibits insulin receptor signalling by decreasing the insulin receptor autophosphorylation and subsequent phosphorylation of insulin receptor substrate-1<sup>39, 40</sup>. In this study we could not confirm the correlations between insulin resistance-related metabolic parameters and serum TNF $\alpha$  concentration. Serum TNF $\alpha$  concentrations in healthy prepubertal children are very low and seem not to participate significantly in regulating insulin and metabolic parameters. Only one significant correlation between TNF $\alpha$  and ACL Ig was found. Perhaps higher concentrations accompanying adiposity are needed for the expression of the above mentioned role of TNF $\alpha$  in constituting more pronounced insulin resistance.

Equally important determinants of insulin sensitivity that interact with insulin signalling pathways such as TNF $\alpha$  and leptin are free fatty acids (FFA) released by adipocytes on activation of hormone sensitive lipase and during lipolysis of TAG-rich lipoproteins by lipoprotein lipase<sup>41, 42</sup>. The fatty acids are taken up by the cells via specific fatty acid transport proteins (fatty acid transport protein, fatty acid binding protein, and others). Most of these functions, including adipocyte differentiation from preadipocytes, are triggered by peroxisome proliferator-activated receptors (in particular PPAR $\gamma_2$ ), that act through transactivation of adipose specific genes, including those that encode for proteins involved in lipid storage and metabolism<sup>43</sup>. PPAR $\gamma_2$  gene expression was increased as a function of BMI in obese humans and decreased in patients losing weight<sup>44</sup>. FFA are probably involved in the pathogenesis of obesity, acting through the induction of two important genes: coding PPAR $\gamma_2$  and coding leptin<sup>45</sup>.

From the group of lipid metabolism regulatory factors, that are under control of PPAR transcription factors, we were able to investigate only the heart fraction of fatty acid binding protein (hFABP). This is one of the membrane long chain fatty acid (LCFA) transporters (along with fatty acid transport protein, fatty acid translocase, and others), that facilitate a quick transfer of LCFA into the muscle cells during increased muscle cell activity, and are increased in hyperlipemias caused by diabetes<sup>46</sup>. In our study, some role of hFABP in explaining serum C-peptid concentration could be suspected

from results of multiple regression analysis (Table 5), along with a significant role of triacylglycerols. These two factors could be more closely connected with a raise of  $\beta$ -cells insulin secretion than other factors studied.

Antibodies against cardiolipin (ACL), a phospholipid localized exclusively in the mitochondria of mammalian cells, are increased in the so called antiphospholipid syndrome, associated with thrombosis and atherosclerosis<sup>47</sup>. The hypothesis was established, that these antibodies are directed not only toward endothelial cells, monocytes, and thrombocytes, but also toward phospholipids and oxidized lipoproteins of LDL and HDL, which could explain some early mechanisms of atherosclerosis<sup>48</sup>. In 50-year-old men, raised levels of both IgG and IgA ACL were positively correlated with the incidence of myocardial infarction 10 to 20 years later<sup>49</sup>. High levels of ACL may be involved in vascular complications of type 1 diabetes mellitus. In our study, multiple regression analysis showed a certain dependence of HOMA IR and intact proinsulin on the serum concentration of ACL-IgG.

In conclusion: Most of the parameters studied, i.e.: a) HOMA IR, derived from fasting serum concentrations of glucose and insulin, b) serum C-peptide and intact proinsulin concentration, c) metabolic events associated with insulin resistance, seem to depend primarily on serum leptin concentration, which is in turn highly correlated with body mass index and inversely correlated with leptin receptor serum concentration. In this group of healthy prepubertal children the serum leptin concentrations contribute to insulin resistance and insulin resistance-related metabolic changes. The role of other factors studied was mostly not significant or absent. TNF $\alpha$  did not influence insulin and metabolic parameters with exception of hFABP. But the role of hFABP and of ACL IgG in regulating insulin sensitivity and insulin secretion seems to be not dominant.

The possibly role of all these factors in the pathogenesis, diagnosis and prognosis of marked insulin-resistance in obese children of comparable age will be the aim of our next study.

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