Obesity, insulin resistance and fatty acid levels in Italian children: 
Relation with +276G>T Adiponectin polymorphism and Pro12Ala PPAR-γ polymorphism

Ana Domingas Manso

Instituto Superior Técnico, Lisbon, Portugal and San Paolo Hospital, Milan, Italy

Abstract
The adiponectin polymorphism was related with insulin resistance on an obese adult population but it is also verified that this correlation varies geographically. Studies focused on the Italian adult obese population are now available but those dedicated to Italian obese children were still missing when this study was started. This study was dedicated to a sample of 93 obese children from northern Italy, between 8 and 13 years old which are followed at San Paolo Hospital in Milan. The results had shown the same trend verified in the adult population: the polymorphism increases insulin resistance (p=0.045 for HOMA*) and it also interferes with LCPUFAs (Long Chain Polyunsaturated Fatty acids) distribution (p values always lower than 0.05).

The PPAR-γ polymorphism was already proved to exert protection against insulin resistance in Italian obese children. However, results of the studies carried out in other countries are controversial suggesting a possible dependency on diet habits. In the present study it was examined PPAR-γ polymorphism incidence and the putative insulin resistance protective effect on a PKU (Phenylketonuria) children population. The PKU population is considered normal (in genetic terms) in which concerns obesity incidence and it exhibits a very important characteristic: the diet is under control. It was then possible to establish a possible relationship between PPAR-γ polymorphism, obesity incidence and the protective effect on insulin resistance without the environmental random of diet habits. This study’s sample population was made of 22 PKU children aged between 8 and 13 years old, also from Northern Italy, that are followed at San Paolo Hospital. However, the results are not conclusive because the population examined is very small and only two children with mutations were found. It is therefore recommended to continue this study in order to increase the size of the sample examined.

Keywords: Obesity, Adiponectin, PPAR-γ, Polymorphisms, Fatty Acids, Insulin Resistance.

* measure of insulin resistance – Homeostatic Model Assessment

Mostly in developed countries, Childhood Obesity is already an increasing problem of public health, being also considered a new epidemiology of the modern society. Recent studies, focused on children and adolescents have shown that it is growing too fast in many countries. That reveals the need to act urgently by adopting wide range and effective strategies to reverse the trend in overweight and obesity and prevent chronic diseases as diabetes type 2 and heart disease. The concern is not only with future adults with health problems but mostly because the young people themselves begin to develop “diseases of old age”. The fatty acids correlation with metabolic syndrome has already been the target for a high number of studies. However, in children there are not so many studies made, especially studies that correlate PUFAs profiles and adiposity and the existent ones show contradictory results (1,2,3). As skeletal muscle is a major site of insulin action. It has been discussed that insulin resistance could be related to phospholipid’s fatty acids’ composition of muscle membranes. The less unsaturated muscle
membranes in children whose mothers have higher fasting insulin and triglycerides levels may as well reflect a genetic reluctance to incorporate PUFAs into membranes, predisposing them to insulin resistance syndromes. (4) It has been demonstrated a metabolic association between low adiponectin levels and dyslipidemia (5). And it has also been shown an inverse correlation between adiponectin levels and pressure levels. (6) However, at this point the most interesting association is the one between adiponectin and insulin resistance. Low levels of adiponectin have been found in patients with insulin resistance (7) as well as with other diseases correlated to this one – Polycystic ovary syndrome (POCS) (8), lipodystrophy (9), NAFLD (10) and HIV (Human Immunodeficiency Virus) (11).

In 2002, Hara et al have suggested an association between +276G>T polymorphism and development of type 2 diabetes (12). More recently, in 2005, Gonzalez et al (13) studied an adult population that revealed a bigger probability to develop glucidic intolerance in condition of homozygotic for +276G>T polymorphism, that is characterized for lower levels of adiponectin in circulation. In another study (14) this polymorphism has been also associated to the risk of developing type 2 diabetes. In other studies (15) it has been demonstrated that this polymorphism is associated to a lower loss of weight in obese adults at dietetic-behavior therapy. It has been proved that in patients with this polymorphism there is a lower increase of adiponectin and a lower decrease of insulin and HOMA values after the dietetic treatment. Seok-Kang et al (16) have demonstrated that also in patients under pharmacologic treatment for type 2 diabetes, the increase of glycemia level is higher in patients without this polymorphism. However in children evidence of this association between this polymorphism and glucidic metabolism is still missing.

PPAR-γ is involved in the metabolic disposal of adipose tissue including adipocyte differentiation, adipogenesis and the pathogenesis of insulin resistance states. The polyunsaturated fatty acids are able to activate PPAR-γ leading to a decrease insulin resistance status. PUFA enriched diets have been associated with increase insulin sensitivity and decrease triglyceride levels in rodents and humans. Arachidonic acid and docosahexaenoic acids are effective PPAR-γ activators. (17)

Material and Methods

The study group for Adiponectin was composed by 93 obese children with ages between 8 and 13. 49 were boys and 44 girls. For PPAR-γ the study group was 22 PKU children with ages between 8 and 13, 12 girls and 10 boys. The criteria chosen to include the selected children were: Weight at moment of birth >= 2500 gestation age between 37 and 42 weeks, no existence of twins, no existence of illness at newborn period, no existence of congenital malformations at newborn period. The exclusion criteria were at least one parent affected by a congenital and/or degenerative metabolic pathology, habitual intake of medicines from one of the parents, levels of triglicemia and colesterolemia above or below the normal. The parents of the children under study had signed a document allowing the tests and also explaining every procedure. The definition of obese children was made according to IOTF’s classification. There have been made anthropometric measurements according to the standard procedure. The BMI was calculated like it was already explained, using the I.S. units - kg/m². Parents’ BMI was also measured and defined as overweight, obese or non-obese according to the BMI results, and according to the standards concepts.

The BMI z-score was calculated using the LMS method, by Cole (18) and two pediatricians had evaluated the Tanner distribution stage – the stage of puberty development. The blood samples were collected in fasting, to evaluate the glucic-insulinemic metabolism index, lipidic structure, plasma fatty acids profile and adiponectin polymorphism. For this study the samples arrived from Pediatric Clinic, from San Paolo’s Hospital, to Fatty Acids and Molecular Biology Laboratories (from the same Hospital). At the first laboratory, the blood arrives in collecting tubes with anti-coagulant (Sodium Citrate, 15%) and, after, they are centrifuge at 4000 rpm, for 10 minutes and the plasma must be taken and conserved at -20°C. At Molecular Biology’s laboratory, it arrives in collecting tubes with anti-coagulant EDTA (EthyleneDiamineTetraCetic acid) and it is conserved as entire blood, in eppendorf tubes, at -20°C, also. Insulin resistance has been evaluated using homeostatic model assessment. Children’s food intake habits have been evaluated by a Questionnaire of Nutrition Habits, according to child’s age and with 116 questions (19). This evaluation was made through a program developed at Pediatric Clinic of San Paolo’s Hospital.
Paolo’s Hospital and based on “Food and Nutrient Data Base”, from Italian National Institute of Nutrition (106).

To evaluate hepatic hyperchogenicity degree, an expert radiologist had done a hepatic abdominal ultrasound with Hitachi H21 (Hitachi High Technology Corporation Ltd, Tokyo Japan), using a conversion of 3,5 MHz. Hepatic echogenicity was evaluated by video registration, independently by the three radiologists, who didn’t know the patients and the results were established consensually.

For PPAR-γ study the criteria followed, once the target is to study also the obesity incidence, were the same used to adiponectin’s experiment. However instead of the questionnaire, the population was all under a controlled diet.

**Adiponectin G276T polymorphism analyses**

To analyze this polymorphism, the DNA of the patients’ blood samples must be extracted, amplified and sequenced. DNA was extracted from samples of blood that had been conserved in a freezer at -20°C. For that it had been used a commercial DNA extraction Kit – QIAmp® DNA Blood Mini Kit, from QIAGEN.

**DNA extraction:**

Defrost one sample of blood, add 200 µl of unfrozen blood and 20 µl of Protease K into an eppendorf tube, add 200 µl of buffer AL, and put in vortex for 15 seconds, incubate in water, at 56°C for 10 minutes, in a thermo regulated bath BE-123T from BICASA Add 200 µl of ethanol (90%) - prepared from 96% Ethanol (0,805 g/mL) from J.T.Baker - and mix at the vortex for 15 seconds With a non graduate pipette remove all the blood to the kit’s columns. Put in centrifuge (Beckman GS-15) at 8000 rpm for 1 minute and after change the tube under the column. Add 500 µl of buffer AW1 and centrifuge at 8000 rpm for 1 minute and after change the tube under the column. Add 500 µl of buffer AW2 and centrifuge at 12500 rpm for 4 minutes and after clean and re-use the tube under the column. Recentrifuge at 12500 rpm for 1 minute and after change the tube under the column to an eppendorf tube. Add 200 µl of buffer AE and centrifuge at 8000 rpm for 1 minute. Conserve eppendorf with the extracted DNA at 4°C

**DNA amplification (Polymerase Chain Reaction):**

Add 5 µl of extracted DNA solution (approximately 170 µg of DNA). Prepare a mix with: 2 µl (80nM) of Primer reverse prepared from a 100µM solution, with a dilution of 1:100, from Invitrogen, 2 µl (80nM) of Primer Forward, prepared from a100µM solution, with a dilution of 1:100, from Invitrogen, 9.8 µl of Water, 4.5 µl of dNTP (Deoxynribonucleotide triphosphate) 100µM, prepared from a solution 556 µM made of 4 solutions, one of each base: GeneAmp® dNTPs (3,2 µmoles, 320 µL, 10mM ) from Applied BioSystems, 1,5 µl (25M) of MgCl₂ co-factor, from a 25mM, 1,5mL solution of Roche, 0,2 µl (1U) of enzyme Taq Polimerase enzyme – AmpliTaq Gold, 250 Units, 5U/ µL, from Roche. Add 20 µl of the mix above. Put the 25 µl into GenAmp PCR System 2400, of Applied Biosystems with the next conditions:- 94°C for 5 minutes; - 94°C for 30 seconds, - 47°C for 30 seconds, - 72°C for 30 seconds, conserve at 4°C until the next step of the process

**Sequencing:**

Doing an asymmetric PCR with primer Forward (once the target gene is next to this border): Add a solution with 3 ml of primer forward, 2 µl of BigDye® Terminator Solution – sequencing RR-100 from Applied Biosystems, 1,5 µl of water and 1,5 µl of buffer BigDye® Terminator, from Applied Biosystems. Add 3 µl of amplified DNA. Put into GenAmp PCR System 2400, of Applied Biosystems with the following conditions: 94°C, 10 seconds; 50°C, 5 seconds; 60°C, 4 min. Column cleaning: centrifuge the resin at 2100 rpm for 3 minutes, put the columns into clean ependorfs, put the 20 µl simples into the columns, centrifuge at 2100 rpm for 3 minutes, Add 7 µl of cleaned DNA, Add 15 µl of water, Run the samples on the sequencing machine – ABI PRISM® 310 Genetic Analyzer, from Applied Biosystems. Read with Sequence Navigator Software

**PPAR-γ polymorphism analyses**

The protocol used is very similar to the one mentioned on point 4.1. So, only the differences will be presented in this point. PCR : Add 5 µl of extracted DNA solution (approximately 170 µg of DNA). Prepare a mix with:2 µl (80nM) of Primer reverse prepared from a 100µM solution, with a dilution of 1:10, from Invitrogen, 2 µl (80nM) of Primer Forward, prepared from a100µM solution, with a dilution of 1:10, from Invitrogen, 9.8 µl of Water, 4,5 µl of dNTP 100µM, prepared from a solution 556 µM made of 4 solutions, one of each base: GeneAmp® dNTPs (3,2 µmoles, 320 µL, 10mM ) from Applied BioSystems, 1,5 µl (25M) of MgCl₂ co-factor, from a 25mM, 1,5mL solution of Roche, 0,2 µl (1U) of enzyme Taq Polimerase enzyme – AmpliTaq Gold, 250 Units, 5U/ µL, from Roche. Add 20 µl of the mix above. Put the 25 µl into GenAmp PCR System 2400, of Applied Biosystems with the next conditions:- 94°C for 10 minutes, - 94°C for 30 seconds, - 56°C for 30
seconds, at 72°C for 30 seconds. Conserve at 4°C
until the next step of the process. 30 cycles

**Plasma Fatty Acids Analyses**

Total Lipid Extraction Protocol – Folch’s method
Put consecutively 1ml of distilled water, 4 ml of
methanol (ISO, MERCK) and 8 ml of chloroform (ACS, ISO, MERCK) into 0.5 ml of plasma. This
should be done in presence of BHT (Butyl-Hydroxi-Toluene) – 5 µl for each ml of mix - which
acts like an antioxidant. Agitate the tubes and leave them at -20ºC for at least 2 hours so
they can separate better their aqueous and organic phase. The phase below – non polar -
must be recovered and dried under N2 stream
and put on a volume of 500 µl of chloroform/methanol (2:1), for homogenization.
To get the total lipids quantity use 25 µl of the
plasma sample to do its gravimetric analysis, with
a microanalitic scale Sartorius M2P.

Protection by methylation
A part of the sample should now be submitted to
methylation so the acid group can be protected
and identified latter. Put 300 µg of total lipids with
3 mL of methanol/HCl 3N (Supelco); Put the tube
in a stove, at 90ºC, for 1 hour; As an intern
standard for quantitative analysis it is done: 10
µg of C17:0 (Sigma) in 100 µl of cycle hexane
(Lichorosolv Merck). The HCl determines with an
acid hydrolysis the ester bounds in which the
groups –COOH of the fatty acids are bounded.
Methanol on the other side, allow the formation of
methyl esters of fatty acids. This transformation
makes the acids more thermo-stable and so
allows a more efficient separation by gas-
chromatography. After the methylation, the
methyl ester (FAME) should be separated from
the rest of the sample.

Extraction of methyl ester groups
Put 6 mL of water in the sample and for three
times put 3 mL of n-hexane. The methyl esters
are recuperated in the superior phase, the
organic one. The organic phase is dried under
nitrogen and recuperated in 100 µl of n-hexane.
At this point the sample is analyzed in a
chromatography capillar (HRGC serie Mega 2
Fisons with FID - Flame ionizing detector).
The separation of the peaks that correspond to each
long chain fatty acid (between 14 and 24 carbon
atoms) is done in capillary column Omegawax
320 (30m, 0.32mm I.D., 0.25µm film thickness.
The temperature gradients used were: Between
60 and 150°C at 10°C/min; between 150 and
170°C at 5°C/min between 170 and 230°C at
2°C/min.

The mobile phase is made of inert gas – helium.
The methyl esters are injected in solution in
evaporation camera of gas chromatograph. Then,
it is transformed in vapour and absorbed by the
stationary phase on the chromatography column.
The speed in the column is lower the heavier the
fatty chain is and the higher the number of double
bounds presents in the same chain. The revelation
of the results is done by the Flame ionizing
detector. This revelator is destructive because the
molecules are broken in the middle by a flame, fed
by air’s hydrogen. The combustion of organic
compounds leads to ions formation that, if
submitted to an electric field, origin an electric
current proportional to quantity of fatty acids.
The recognization of the methylesters groups is done
by comparision between retention times of
chromatographic peaks contained in the sample
and retention times of methyl ester of a standard
solution (SIGMA, cod.189-19, with methyl esters
saturated, monoinsaturated and polinsaturated
with chain’s length between 8 and 24 carbon
atoms).

**Peaks integration**
This integration is done by an appropriated
software - Chromcard versione 1.19.

**Results**
The discrete random variables are
expressed in terms of average or number of
observed cases (percentage). For continue
random variables ANOVA or appropriate
Mann-Whitney tests were used.
The triglycerides, basal insulin and HOMA
that do not present a Gaussian distribution,
were log-transformed in order to evaluate
the relationship with the polymorphism.
For BMI, because directly it influences
insulin resistance corrections with z-score
calculation were made.

The difference between averages were
considered statistically significant for values
p < 0.05. In cases of significance (P<0,05) –
only for adiponectin’s case - association
between polymorphism, insulin resistance
(basal insulin and HOMA) and plasma fatty
acids values was repeatedly tested with
logistic multiple regression.
This analysis was made using in SPSS (Statistical Package for the Social Sciences) 15.0 for windows.

**Adiponectin polymorphism**

Evaluate the frequency of the +276G<T adiponectin polymorphism in obese children population

The allelic frequency of the polymorphism +276G>T on the population under study (n=93) was verified to be 29.6%, as the number of alleles are 186 and the total mutated alleles are 55 (37 from heterozygotes and 18 from homozygotes). The polymorphism distribution verified is:

- n=47, or 50.5% of the patients do not present polymorphism
- n=37, or 39.8% of the patients present heterozygote polymorphism
- n=9, or 9.7% of the patients presents present homozygote polymorphism

**Anthropometric parameters and children metabolic profile, according to existence of +276G>T polymorphism**

Table 5.1 represents the occurrence of polymorphism on children under study and the anthropometric parameters.

The distribution verified between genders does not represent significant differences. Therefore it cannot be said that one polymorphism is more common in one gender than in another.

Concerning glucidic metabolism a decrease of insulinemia in fasting (p=0.007) and HOMA (p=0.006) was found when comparing obese children with polymorphism and without. This relationship is relevant also when the factors from which these two items depend are normalized: weight, BMI z score, years of obesity and gender.

For anthropometric indexes and metabolic profile there were not observed any relevant differences, particularly there is no association between BMI z score and polymorphism +276G>T presence.

### Table 5.2. – Averages and p-value for anthropologic and metabolic data distributed according to SNP+276G-T occurrence

<table>
<thead>
<tr>
<th></th>
<th>Snp +276 G/G (n= 47)</th>
<th>Snp +276 G/T e T/T (n= 46)</th>
<th>P-value ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>24/23</td>
<td>25/21</td>
<td>0.751</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109 (10)</td>
<td>110 (10)</td>
<td>0.568</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68 (8)</td>
<td>67 (7)</td>
<td>0.487</td>
</tr>
<tr>
<td>Age</td>
<td>9.97 (1.66)</td>
<td>10.38 (1.82)</td>
<td>0.240</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>2.16 (0.41)</td>
<td>2.28 (0.36)</td>
<td>0.089</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>85.38 (5.7)</td>
<td>87.73 (9.3)</td>
<td>0.225</td>
</tr>
<tr>
<td>Insulinemia (mU/ml)</td>
<td>12.23 (6.4)</td>
<td>18.5 (7.4)</td>
<td>0.061*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.57 (1.4)</td>
<td>4.04 (1.7)</td>
<td>0.045*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>170.04 (29.7)</td>
<td>168.67 (25)</td>
<td>0.812</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>49.79 (14.1)</td>
<td>48.78 (10.1)</td>
<td>0.222</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>98.45 (30)</td>
<td>95.41 (25)</td>
<td>0.900</td>
</tr>
<tr>
<td>HDL/LDL</td>
<td>2.11 (0.86)</td>
<td>2.02 (0.66)</td>
<td>0.380</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>109.98 (64.6)</td>
<td>108.93 (56.6)</td>
<td>0.540</td>
</tr>
</tbody>
</table>

**Fatty Acids and phospholipid’s profile in obese children, according to the presence of +276G>T polymorphism**

Tables 5.4 and 5.5 show the fatty acids profile in plasma and in phospholipids divided as presence or absence of adiponectin’s polymorphism.

It can be observed that the plasma profiles of children with polymorphism have higher levels of saturated fat (p=0.056), of n-6 LCPUFAs (p=0.015) and of arachidonic acid (0,011) and lower levels of monounsaturated (p=0.016) and alpha-linolenic acid (p=0.011)

Phospholipid’s profiles of children with polymorphism have evidenced higher values of n-6/n-3 LCPUFAs levels (p=0.005) and lower levels of n-3 LCPUFAs (0,043), of alpha-linolenic acid (p=0.017) and DHA/AA (p=0.048).
These associations are relevant also when standardized with BMI z-score correction.

A logistic regression model was used in order evidence any independent association between polymorphism and HOMA and % of plasma phospholipids. This analysis has shown that HOMA (OR 1.35 CI 1.03 – 1.77), monounsaturated levels (OR 1.46 CI 1.13 – 1.87) and ratio n-6/n-3 LCPUFAs (OR 3.4 CI 1.63 – 7.1 ), are independently associated to adiponectin’s polymorphism.

Hepatic echogenicity in mutated children and respective fatty acid and phospholipid profile

For hepatic echogenicity it was observed that the patients with polymorphism shown a higher frequency of this condition than the ones that do not have the polymorphism (p=0.063). There was also found some relevance on the distribution of hepatic hyperechogenicity degree and presence of polymorphism (p=0.042), like it can be seen in the table 5.6.
Table 5.6 – Distribution of Hepatic echogenicity degree according to polymorphism’s occurrence

<table>
<thead>
<tr>
<th>Degree</th>
<th>Polymorphism</th>
<th>Without polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>57.8</td>
<td>76.1</td>
</tr>
<tr>
<td>1</td>
<td>24.4</td>
<td>19.6</td>
</tr>
<tr>
<td>2</td>
<td>13.3</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>4.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>

In the table 5.7 are the results of the confrontation between fatty acids profile in phospholipids in patients with polymorphism (n=46) divided according to the presence or absence of hepatic hyperechogenicity. Children with hepatic hyperechogenicity have higher levels of n-6 LCPUFAs (p=0.05) in plasma phospholipids.

Table 5.7 – Averages and p-values for Phospholipid’s Fatty Acids’ profile according to presence or absence of Steatosis

<table>
<thead>
<tr>
<th>Snp</th>
<th>+276</th>
<th>+276</th>
<th>G/G</th>
<th>G/T</th>
<th>e</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>47.9</td>
<td>49.1</td>
<td>0.682</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>16.0</td>
<td>17</td>
<td>0.189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polynomials</td>
<td>36.1</td>
<td>34.9</td>
<td>0.196</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6 PUFA</td>
<td>32.8</td>
<td>31.9</td>
<td>0.189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.401</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6/n-3 PUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6/n-3 LCPUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>19.8</td>
<td>18.6</td>
<td>0.116</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>8.7</td>
<td>9.1</td>
<td>0.767</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-3 LCPUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-3 LCPUFA</td>
<td>3.1</td>
<td>2.9</td>
<td>0.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.12</td>
<td>0.09</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>0.39</td>
<td>0.32</td>
<td>0.037</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>0.39</td>
<td>0.32</td>
<td>0.123</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>2.0</td>
<td>1.9</td>
<td>0.611</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:3n-9</td>
<td>0.14</td>
<td>0.17</td>
<td>0.899</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5n-3/3/C20:4n-6</td>
<td>0.04</td>
<td>0.03</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:6n-3/3/C20:4n-6</td>
<td>0.24</td>
<td>0.22</td>
<td>0.328</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:6n-3/C18:3n-3</td>
<td>18.6</td>
<td>23.0</td>
<td>0.171</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8 shows the relationship between fatty acids profile and every patients with hepatic hyperechogenicity (n=30) stratified according to the presence or absence of polymorphism. In patients without hyperechogenicity the polymorphism is associated to lower levels of alpha-linolenic acid (0.037) and lower ratio EPA/AA (p=0.007).
**PPAR-γ Results**

Evaluate the frequency of the PPAR-γ polymorphism in PKU children population.

The allelic frequency of this polymorphism, in the studied PKU population \( n=22 \) was verified to be 4.5%, as the number of alleles are 44 and the total mutated alleles are 2 (because both mutations are heterozygotes). The observed polymorphism distribution is:

- \( n=20 \), or 91% of the patients do not present polymorphism
- \( n=2 \), or 9% of the patients present heterozygote polymorphism

Anthropometric parameters and children metabolic profile, according to existence of PPAR-γ polymorphism

In this case, there is, besides a non anthropologic association (as p value for BMI z-score is 0.308) with the presence of the polymorphism, there is not a metabolic association either (p values for insulinemia and HOMA are respectively 0.843 and 0.771).

**Table 5.9** - Averages and p-value for anthropologic and metabolic data distributed according to PPAR-γ polymorphism occurrence

<table>
<thead>
<tr>
<th>Without polymorphism</th>
<th>Polymorphism</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=20</td>
<td>n=2</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(boys/girls)</td>
<td>9/11</td>
<td>1/1</td>
</tr>
<tr>
<td>Age</td>
<td>9.1 (1.7)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>0.4817 (1.3)</td>
<td>1.4 (0.014)</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>83 (6.1)</td>
<td>81.5 (0.71)</td>
</tr>
<tr>
<td>Insulinemia µU/ml</td>
<td>7.4 (4.5)</td>
<td>8.1 (4.0)</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.5 (0.85)</td>
<td>1.6 (0.8)</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>140.5 (22.6)</td>
<td>159.5 (6.3)</td>
</tr>
</tbody>
</table>

**Discussion and Conclusions**

Adiponectin polymorphism results

The present study indicates a SNP +276G>T presence frequency in about 50% of the obese sample studied. This frequency is in line with the ones already presented in other studies.

The results evidence a possible relationship between an increase of insulin sensitivity and
presence of SNP+276. As is can be seen on table 5.2, children with this polymorphism show higher values of insulinemia in fasting and also of HOMA. This trend was the same found already in Italian adult population (20).

Red blood cell profile seems to reflect the Fatty Acids profile (and most of all PUFAs) of membrane’s phospholipids of muscular cells. It has been already demonstrated that the type of diet is equally relevant on erythrocytes and muscular cells’ fatty acids’ composition. (21)

It is known that an increase in n-6/n-3 LCPUFAs in muscular cells’ membrane is normally associated to insulin resistance. Since relevant differences on consumption of the principal macronutrients were not found, it can be hypothesized that the presence of SNP +276 G>T can induce a change on LCPUFAs’ distribution of muscular cells. This change can then expose the polymorphism’s carriers to a higher risk of developing insulin resistance condition.

One of the major limitations of this study is the impossibility to estimate the dietary consumption of every single fatty acid and so evaluate the relationship between that consumption and plasma fatty acids’ levels.

Considering this limitation, it can also be concluded that obese children that have polymorphism SNP +276 G>T on adiponectin’s gene seem to be more exposed to metabolic conditions through a mechanism that includes a change on LCPUFAs distribution at muscular cells’ membrane level.

PPAR-γ polymorphism results

San Paolo Hospital, in Milan, is the principal Italian center for metabolic diseases, in particular for PKU is the reference center. PKU screening has been mandatory since 1992. Since then all the patients with PKU at neonatal screening are sent to this hospital. So the PKU population here is especially large (approximately 550). As an under-control diet population it is an opportunity to study some evidences already found on obese children and it can be conclude how relevant the genetic predisposition or protection is once the principal environmental conditions are now under controlled.

Not all the PKU children are under diet. In fact there is a softer type of PKU that must only be continually followed by a doctor. The first step was to select a population under diet, and so with the correspondent mutations of genotypes 1 and 2 for PHA mutations (table 1.3). These children have been accompanied by a nutritionist since very young ages. And although the principal concern is the decrease of the consumption of phenylalanine, the nutritionist role becomes also to give to these children a balanced diet on every macronutrient. Another concern when selecting the study group is the data available. This is a very rich population in terms of medical data, however they are not an obese population and so the data available specifically for this study is not as complete as the one available for adiponectin’s study. The principal concern was to have levels of insulinemia and glycemia (and so HOMA can be calculated) and some phospholipid’s fatty acids, once these were the values that, on the previous study made on obese children population (17), presented one association with PPAR-γ polymorphism.

A previous study made in this hospital (17), revealed an association (protective effect) between PPAR-γ polymorphism and insulin resistance on obese children. Conclusions were that it acts preventing insulin resistance – one of the worst metabolic conditions associated to obesity. However some other studies, made in different countries like Brazil and France lead to different conclusions, suggesting a dependency on location and so, maybe, on diet habits. Then, the present study intends to give an idea on how much of the associations done in past studies are actually relevant comparing with food intake habits relevancy.

The biggest limitation of this study was the number of patients studied – only 22 and the fact that, in these 22, only 2 are mutated on PPAR-γ. This low number happened because the blood tests are made only when these children go to “day hospital” (a
day per year when they should go to the hospital to be controlled). In the future this number should definitely be increased. Another limitation is, as they are an obesity independent population, there are no data available on hepatic hyperechogenicity, as there is no sense on doing an ultrasound to these children. However, the data available should be enough to understand if there is a relevant real relationship between obesity occurrence and PPAR-γ polymorphism.

In fact, only two of these 22 children have the polymorphism. And for that reason the results cannot be conclusive. But the fact is that there weren’t found no differences between these two mutated children and the others in which concerns the insulin resistance or higher PUFAs levels. But, they are just two mutated cases…

However there is a situation of a child, with no polymorphism, that presents high levels of insulinemia (23.7 µU/ml) and HOMA (4.4), which indicates trend to insulin resistance, suggesting another genetic predisposition to obesity once the diet is for itself very well controlled, but without the protective effect of PPAR-γ Pro12Ala polymorphism.

The results are not conclusive also because there was not a significant correlation with relevance between the parameters in study and polymorphism’s incidence (all p values were inferior to 0.05 – as can be seen on table 5.10). If there were more cases like the one described – of a wild type carrier with more tendency to insulin resistance - and the number of heterozygotes found increased but continue to reveal no tendency to insulin resistance especially if some demonstrate tendency to obesity (with higher z-score BMI, for instance), it could be hypothesized that aside from the type of diet there is a real association with PPAR-γ and obesity incidence and also that once happened has a protective effect for insulin resistance. But about 10% of mutation frequency is a too low value, especially when the total population is so small has 22 patients.

Particularly about the two studies realized some considerations can be made: For Adiponectin it is suggested a new study, similar to the one presented in this work for PPAR-g. It should be study the impact of the results on a sample of population under controlled diet and analyze if the correlation found between the polymorphism 276G>T and Insulin resistance and LCPUFAs has the same significance, or in the other hand if the diet affects it. For PPAR-g study should be continued in order to increase the population and find more mutated cases and therefore some conclusions can be drawn.

**Bibliographic references**

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20 S.A.Ritchiem, The link between abdominal obesity, metabolic syndrome and cardiovascular disease, Science Direct, November 2006