Hepatic Fat Accumulation Is Modulated by the Interaction between the rs738409 Variant in the PNPLA3 Gene and the Dietary Omega6/Omega3 PUFA Intake

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Abstract

Background: A single nucleotide polymorphism (SNP), the rs738409, in the patatin like phospholipase 3 gene (PNPLA3) has been recently associated with increased hepatic steatosis and ALT levels in adults and children. Given the potential role of PNPLA3 in fatty liver development, we aimed to explore whether the influence of PNPLA3 genotype on hepatic fat in obese youth might be modulated by dietary factors such as essential omega polyunsaturated fatty acids (PUFA) intake.

Materials and Methods: We studied 127 children and adolescents (56 boys, 71 girls; 58 Caucasians; 30 African Americans and 39 Hispanics; mean age 14.7 ± 3.3; mean BMI 30.7 ± 7.2). The dietary composition was assessed by the Nutrition Data System for Research (NDS-R version 2011). The patients underwent a MRI study to assess the liver fat content (HFF%), ALT measurement and the genotyping of the rs738409 SNP by automatic sequencing.

Results: As previously observed, HFF% and ALT levels varied according to the genotype in each ethnicity. ALT levels and HFF% were significantly influenced by the interaction between genotype and omega-6/omega-3 PUFA ratio (n-6/n-3), p = 0.003 and p = 0.002, respectively. HFF% and ALT levels were, in fact, related to the n-6/n-3 consumption only in subjects homozygote for the G allele of the rs738409 (r2 = 0.45, p = 0.001 and r2 = 0.40, p = 0.006, respectively).

Conclusions: These findings suggest that the association of a high dietary n-6/n-3 PUFA with fatty liver and liver damage in obese youths may be driven by a predisposing genotype.

Recently two studies have started pinpointing the physiologic role of PNPLA3 and how this is affected by the rs738409 minor allele [10,11]. In particular, it has been shown that the rs738409 minor allele is associated with a reduced hydrolytic capability of the protein [10] and that free fatty acids (FFA) potentiate the effect of PNPLA3 rs738409 variant on intra-hepatic triglycerides accumulation [11]. This latter observation is of particular interest if we consider that the amount and the type of FFAs provided by the diet seem to play a pivotal role in the development of NAFLD [12]. Recent literature provides clues that the dietary imbalance between omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) leads to development of an adverse cardiovascular and metabolic profile and contribute to the pathogenesis of NAFLD [13]. Biochemical analyses have shown alteration in the hepatic long chain fatty acid composition toward an increase in the n-6/n-3 PUFA ratio [14] and animal studies demonstrated that an excess of n-6 PUFA in the liver is associated with a pro-inflammatory state [15,16] and an increased lipogenesis leading to massive fat accumulation into the liver [17–19]. Given these
Conducted according to the principles expressed in the Declaration from all participants. All clinical investigations have been parental informed consent and written child assent were obtained by the Yale University Human Investigation Committee. Written glucose tolerance (NGT), 27 (21%) impaired glucose tolerance (IGT) and 3 (3%) type 2 diabetes (T2D). The study was approved previously described [3] revealed that 97 (76%) had a normal prevalence. Prior to analyze the data all the variables were tested for normality, with non-normally distributed variables log transformed to be better approximated by normality, except for HFF% for which a square root transformation was used. Differences in anthropometric features and in dietary intake among the groups of genotype were tested by ANOVA; age, gender, z-score BMI and ethnicity were used as covariates when appropriate.

Since the primary aim of the study was to explore whether the interaction between the rs738409 variant and n-6/n-3 PUFA could modulate hepatic fat accumulation in obese children and adolescents, the primary outcome of the study was the HFF%. In order to explore whether an eventual interaction may also drive to liver damage we tested as secondary outcome the ALT levels. ALT values were not available for 9 subjects (4 CC, 3 CG and 2 GG). To test the interaction between the genotype and dietary n-6/n-3 PUFA on the HFF% and ALT levels the subjects of the three ethnic groups were pooled, a regression coefficient (\( r^2 \)) was used and the genotype was coded with 0, 1, or 2 corresponding to the number of minor alleles carried by each individual. An interaction term between the rs738409 and the dietary intake of n-6/n-3 PUFA was added in a regression model in which age, gender, ethnicity, z-score BMI and glucose tolerance were used as covariates. Unless otherwise specified, for all the data raw means and standard deviations are shown.

Results

The frequency of the minor allele for the rs738409 was 0.26 in Caucasians, 0.20 in African Americans and 0.49 in Hispanics (p = 0.01). The allele frequencies were consistent with those shown in similar ethnic groups in the Allele Frequency Database (ALFRED, http://alfred.med.yale.edu) as well as in HAPMAP (http://hapmap.ncbi.nlm.nih.gov/). Within each ethnic group there was no evidence against the null hypothesis that the genotype distribution was in Hardy Weinberg equilibrium for all of the variants (all p>0.05).

Table 1 shows the clinical features of the study population according to the genotype in each ethnic group. The three groups of genotype did not differ in terms of age, gender, z-score BMI and glucose tolerance in each ethnic group. As previously observed, HFF% varied among the PNPLA3 rs738409 groups of genotype (Caucasians p = 0.0006, African Americans p = 0.001, Hispanics p = 0.09) [3], and there was a trend toward increased ALT levels in subjects homozygous for the G allele.

In the whole population the mean caloric intake was 2377.7±580.9 Kcal, the mean intake of n-3 PUFA was 1.8±1.1 grams and the mean intake of n-6 PUFA was 14.8±7.9 grams, the mean dietary n-6/n-3 PUFA was 8.9±2.4. There was no difference among the genotypes for the energy intake (CC 2351.3±537.0 Kcal, CG 2393.6±553.0 Kcal, GG 2444±693.8 Kcal, p = 0.82), n-6 PUFA intake (CC 15.5±0.7 grams, CG 14.3±6.3 grams, GG 13.4±7.9 grams,
p = 0.51) and n-3 PUFA intake (CC 1.9±1.3 grams, CG 1.9±0.8 grams, GG 1.4±0.7 grams, p = 0.26). The mean dietary n-6/n-3 PUFA was 9.56±5.26 in CC, 7.84±2.9 in CG and 9.09±2.3 in GG (p = 0.10).

We observed an interaction between the PNPLA3 gene variant and the n-6/n-3 PUFA in influencing HFF% (p = 0.002) and ALT levels (p = 0.003), independently of age, gender, z-score BMI, ethnicity and glucose tolerance (p = 0.017 and p = 0.016 respectively). This interaction was the result of a different regression degree between the n-6/n-3 PUFA and HFF% or ALT in the groups of genotype. In fact, there was a strong association in the GG group between n-6/n-3 PUFA and ALT or HFF% (r^2 = 0.40, p = 0.006 and r^2 = 0.45, p = 0.001 respectively), while the same association was not present in the other two groups of genotype (CC and CG) (figure 1 and figure 2).

Discussion

The main result of this study is the observation of an interaction between the PNPLA3 rs738409 and n-6/n-3 PUFA on hepatic fat content and ALT levels. This novel finding helps to explain the strong association between the rs738409 SNP and NAFLD that has been largely and repeatedly demonstrated [2–8] and raises new questions about the function of the PNPLA3 itself as well as

| Table 1. Clinical features of the study population according to ethnicity and PNPLA3 rs738409 genotype. |
|-----------------|-----------------|-----------------|-----------------|------|
| Age (years)    | CC (34)         | CG (18)         | GG (6)          | p-value |
| Gender (M/F) % | 15.68±3.28      | 13.76±3.62      | 13.91±2.47      | 0.12  |
| GT (NGT/IGT/T2D) % | 41/59         | 33/67           | 33/67           | 0.83  |
| Energy intake (Kcal) | 2471.3±622.8   | 2379.3±561.9   | 2162.4±601.2   | 0.78  |
| n-3 PUFA (g) * | 2.2±1.71        | 2.11±0.72       | 1.20±0.43       | 0.14  |
| n-6 PUFA (g) * | 16.8±8.64       | 15.07±6.77      | 11.52±3.63      | 0.59  |
| n-6/n-3 PUFA*  | 9.86±5.92       | 7.64±3.21       | 9.64±9.0        | 0.17  |
| BMI (Kg/m2)    | 29.77±7.47      | 29.10±5.47      | 35.28±6.57      | 0.15  |
| HFF%**         | 4.13±7.23       | 6.58±9.65       | 27.40±13.50     | 0.0006|
| ALT (UI/L)*    | 21.14±14.15     | 36.73±48.25     | 32.80±17.48     | 0.33  |

| AFRICAN AMERICANS |
|-----------------|-----------------|-----------------|-----------------|------|
| Age (years)    | CC (19)         | CG (10)         | GG (1)          | p-value |
| Gender (M/F) % | 16.57±3.38      | 16.18±3.13      | 12.00           | 0.41  |
| GT (NGT/IGT/T2D) % | 37/63         | 40/60           | 0/100           | 0.73  |
| Energy intake (Kcal) | 2358.5±602.8   | 2233.4±333.9   | 2446.5          | 0.77  |
| n-3 PUFA (g) * | 1.69±0.85       | 1.90±1.33       | 1.58            | 0.57  |
| n-6 PUFA (g) * | 14.27±6.64      | 12.83±7.32      | 16.11           | 0.97  |
| BMI (Kg/m2)    | 32.43±7.41      | 31.97±8.49      | 38.19           | 0.15  |
| HFF%**         | 0.65±1.10       | 2.30±3.05       | 39.4            | 0.0001|
| ALT (UI/L)*    | 15.11±6.39      | 13.50±8.05      | 52              | 0.34  |

| HISPANICS      |
|-----------------|-----------------|-----------------|-----------------|------|
| Age (years)    | CC (13)         | CG (14)         | GG (12)         | p-value |
| Gender (M/F) % | 12.97±3.08      | 13.27±1.99      | 14.09±3.34      | 0.33  |
| GT (NGT/IGT/T2D) % | 54/46        | 73/27           | 50/50           | 0.40  |
| Energy intake (Kcal) | 2168.4±344.1   | 2561.3±673.3   | 2572.9±753.5   | 0.31  |
| n-3 PUFA (g) * | 1.60±0.73       | 1.81±0.72       | 1.62±0.94       | 0.73  |
| n-6 PUFA (g) * | 14.29±11.73     | 14.99±5.82      | 14.16±6.96      | 0.69  |
| n-6/n-3 PUFA*  | 8.85±5.15       | 8.65±2.65       | 8.79±2.91       | 0.74  |
| BMI (Kg/m2)    | 31.05±6.19      | 29.39±8.34      | 29.64±5.87      | 0.51  |
| HFF%**         | 8.45±9.40       | 9.96±13.57      | 15.59±13.28     | 0.096 |
| ALT (UI/L)*    | 25.36±15.88     | 29.78±21.75     | 47.18±30.46     | 0.010 |

* = log transformed and adjusted for age, gender, BMI, glucose tolerance. ** = Square root transformed and adjusted for age, gender, BMI, glucose tolerance. GT = glucose tolerance. NGT = normal glucose tolerance, IGT = impaired glucose tolerance, T2D = type 2 diabetes.

doi:10.1371/journal.pone.0037827.t001
about its potential implications in the development of cardiovascular diseases.

Since the association between the PNPLA3 rs738409 and NAFLD has been discovered [2], several studies have tried to unravel the pathogenetic mechanism underlying this association. It has been suggested that this variant may cause a gain of function of the protein, which would act as a lipogenic factor [24]. In fact, while knock out mice for the pnpla3 do not show any increased fat accumulation into the liver with respect to the wild types [25, 26], the administration through viral vectors of the mutated PNPLA3 confers to wild type mice a higher susceptibility to fatty liver [24]. Consistently, it was also shown that SREBP-1c, activated by carbohydrate feeding, transcriptionally activates pnpla3 as well as several genes encoding enzymes in the fatty acid biosynthetic pathway [23]. This mechanism seems also to be indirectly supported by studies showing an interaction between the carbohydrates intake and the PNPLA3 rs738409 in determining the development of fatty liver [9]. The only data that do not fit with this hypothesis is the lack of association of the PNPLA3 variant with increased plasma triglycerides [2, 3].

Our group has previously proposed another mechanistic hypothesis based on the observation that subjects carrying the G allele show smaller subcutaneous adipose cells. Since the PNPLA3 has been suggested to be a potential growth factor for adipose cells [3], we suggested that in these subjects there might be an overflow of free fatty acids from the adipose tissue to the liver given the lower capacity of their adipose cells to store the FFA [3].

*Figure 1. Interaction between PNPLA3 rs738409 and n-6/n-3 PUFA in modulating HFF%.* The figure shows a different degree of regression between HFF% (square root) and n-6/n-3 PUFA (log10) in the three genotypes. In the CC (Panel A) and CG (Panel B) group there was no association between HFF% and n-6/n-3 PUFA (r² = 0.0004, p = 0.86 and r² = 0.018, p = 0.39, respectively). Only in the GG group (Panel C) there was a strong association between HFF% and n-6/n-3 PUFA (r² = 0.45, p = 0.001).

doi:10.1371/journal.pone.0037827.g001

*Figure 2. Interaction between PNPLA3 rs738409 and n-6/n-3 PUFA in modulating ALT levels.* The figure shows a different degree of regression between ALT (log10) and n-6/n-3 PUFA (log10) in the three genotypes. In the CC (Panel A) and CG (Panel B) group there was no association between ALT and n-6/n-3 PUFA (r² = 0.0006, p = 0.91 and r² = 0.015, p = 0.21 respectively). Only in the GG group (Panel C) there was a strong association between HFF% and n-6/n-3 PUFA (r² = 0.40, p = 0.006).

doi:10.1371/journal.pone.0037827.g002
pnpla3 does not contribute significantly to adipose cell development [25].

Perhaps more promising are the results provided by studies focusing on the hydrolytic action of the PNPLA3 given that the PNPLA3 along with the acylglycerol transacylase activity also has a triacylglycerol hydrolase function [10]. By studying this latter mechanism, it has been recently suggested that the rs738409 variant might cause a lack of the PNPLA3 hydrolytic function [10]. In particular, the gene variant seems to lower the protein activity in hydrolizing the n-6 of about 15% [10]. The n-9 represents the most common fatty acids in the diet, deriving from meat, olive oil, sesame oil, almonds, and avocados, but they are also synthesized starting from essential polyunsaturated fatty acids such as the n-6 [27]. More recently, another study by Perttila et al. has shown that the presence of a methionine in position 148 in the PNPLA3 enhances the cellular accumulation of triglycerides in the presence of an excess FFA by significantly slowing down the triglycerides hydrolisys [11]. Our observation seems to be consistent with these animal and in vitro studies [10,11,28] supporting indirectly the role of PNPLA3 in lipid hydrolisys. In fact, given those evidences, one could speculate that the overload of n-6 in the diet will serve both as substrate for new triglycerides and in the meantime will be slowing down or delaying the hydrolytic function of the PNPLA3. Thus, in subjects carrying the rs738409 minor allele, while the newly formed triglycerides will tend to accumulate into the liver leading to hepatic steatosis, the excess of n-6 not incorporated into triglycerides will lead to the over-synthesis of proinflammatory n-6 derived species, which in turn trigger the second hit responsible for the inflammation that leads to NASH.

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Acknowledgments
The authors are grateful to the patients and their families as well as to the Yale Center for Genome Analyses (YCGA) and Yale Center for Clinical Investigation (YCCI) personnel. Nicola Santoro is personally indebted to Silvia Raveria and Tetyana Zayats for their thoughts and suggestions.

Author Contributions
Conceived and designed the experiments: NS SC. Performed the experiments: MS GK KM MMS BP. Analyzed the data: NS MK. Contributed reagents/materials/analysis tools: SC NS KM MMS GK MS. Wrote the paper: SC NS.

Our observation of an interaction between the PNPLA3 rs738409 variant and the high n-6/n-3 PUFA suggests that we could provide a targeted therapy to subjects with NAFLD homozygous for the minor allele either reducing the dietary n-6 amount or alternatively increasing dietary intake of foods rich in n-3 PUFA, such as salmon, tuna, and flaxseed oil, or supplementing the diet with n-3 PUFA. The omega 3 supplementation, in fact, by balancing the n-6/n-3 PUFA has been shown to be effective in reversing hepatic steatosis in animal as well as in humans [14,29]. In conclusion, our findings show an interaction between the PNPLA3 rs738409 variant and the dietary n-6/n-3 PUFA in modulating the hepatic fat accumulation and the liver damage in obese youths. The study findings generate new questions about the function of the PNPLA3 itself and pose new opportunity for targeted therapy in patients with NAFLD.

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