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SNPs of the PPAR-α Gene Influence the Conversion from Impaired Glucose Tolerance to Type 2 Diabetes: the STOP-NIDDM Trial

Short title: *PPARA* polymorphisms in diabetes

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Abstract. Peroxisome proliferator-activated receptor (PPAR) α, a transcription factor of the nuclear receptor superfamily, regulates fatty acid oxidation. We evaluated the association of single nucleotide polymorphisms (SNPs) of the PPAR-α gene (*PPARA*) with the conversion from impaired glucose tolerance (IGT) to type 2 diabetes in 767 subjects of the STOP-NIDDM Trial, aiming to investigate the effect of acarbose compared to placebo on the prevention of diabetes. In the placebo group, the G (162V) allele of rs1800206 increased the risk for diabetes by 1.9-fold (95% confidence intervals (CI), 1.05-3.58) and was associated with elevated levels of plasma glucose and insulin. The effect of this allele on the risk of diabetes in the placebo group was enhanced by the simultaneous presence of the risk alleles of the PPAR-γ2, PPAR-γ coactivator 1α, and hepatic nuclear factor 4α genes (odds ratios 2.2, 2.5 and 3.4, respectively). In the acarbose group, subjects carrying the minor G allele of rs4253776 and the CC genotype of rs4253778 of *PPARA* had a 1.7- and 2.7-fold increased risk for diabetes. Our data indicate that SNPs of *PPARA* increase the risk of type 2 diabetes alone and in combination with the SNPs of other genes, acting closely with PPAR-α.

Key words: Type 2 diabetes; impaired glucose tolerance; PPARA; PPARG; PGC1A; HNF4A; polymorphisms

Abbreviations: PPAR, peroxisome proliferator-activated receptor; PGC-1α , PPAR-γ coactivator 1α; HNF4α, hepatic nuclear factor 4α; SNP, single nucleotide polymorphism; *PPARA*, PPAR-α gene; *PPARG2*, PPAR-γ2 gene; *HNF4A*, HNF4α gene; *PGC1A*, PGC-1α gene; BMI, body mass index; IGT, impaired glucose tolerance; STOP-NIDDM, Study TO Prevent Non Insulin Dependent Diabetes Mellitus; MAF, minor allele frequency; LD, linkage disequilibrium; OR, odds ratio; CI, confidence interval.

Key proteins involved in lipid metabolism are under the transcriptional control of peroxisome proliferator-activated receptor (PPAR) α (1). Among the three PPAR subtypes, PPAR-α is found predominantly in the liver, kidney and heart, where it upregulates the expression of genes involved in fatty acid metabolism (1), particularly when activated by PPAR-γ coactivator 1α (PGC-1α) (2).

PPAR-α agonists lower plasma lipid levels, decrease intrahepatic and skeletal muscle lipid accumulation and adiposity, normalize glucose and insulin concentrations, and therefore markedly reduce insulin resistance and the risk of type 2 diabetes (3-5) in various rodent models of type 2 diabetes and insulin resistance, whereas gemfibrozil and fenofibrate can improve insulin sensitivity in humans (6,7).

Along with regulation of lipid and glucose metabolism PPAR-α is as an attractive candidate gene for type 2 diabetes. Among previous studies (8,9), only one cross-sectional study has reported an association of haplotype of *PPARA* with the age of diabetes diagnosis (10). Therefore, we evaluated the association of SNPs of *PPARA* with the conversion from impaired glucose tolerance (IGT) to type 2 diabetes in subjects of the STOP-NIDDM trial. Furthermore, the effects of SNPs of *PPARA*, along with the SNPs of PPAR-γ coactivator 1α (*PGC-1A*), PPAR-γ2, (*PPARG2*), and hepatic nuclear factor 4α (*HNF4A*) on the conversion to diabetes were investigated.

RESEARCH DESIGN AND METHODS

Subjects and study design. The STOP-NIDDM trial was a double-blind, placebo-controlled study, which randomized 1429 subjects with IGT to either acarbose or placebo group (11). Annual oral glucose tolerance tests were performed to evaluate the conversion to diabetes. The entire population was followed up on the average of 3.3 ± 1.2 years. DNA was available from 767 subjects from 7 countries (385 men and 382 women; 354 treated with acarbose, and 413 with placebo). Their mean BMI was 30.8 ± 4.1 kg/m² and age 54.7 ± 7.9 years. All participants signed an informed consent form, approved by appropriate Institutional Review Boards.

DNA analyses. We screened three SNPs from the study of Flavell DM *et al.* (10) (rs135559, rs1800206 and rs4253778). In addition, eight SNPs were selected using the Tagger software (12) (http://www.broad.mit.edu/mpg/tagger/faq.html) and data available from HapMap project (13) (http://www.hapmap.org; Data Release#20/phase II, January 2006). In the genomic region of 100 kilo-base pairs (kbp) including 93.2-kbp *PPARA* locus, 10 of all 11 selected SNPs captured up to 70% of common variants with minor allele frequency (MAF)>5%; r^2>0.8 (rs135539 was not genotyped in HapMap).

Screening of SNPs of *PPARA* was performed with the TaqMan Allelic Discrimination Assays (Applied Biosystems). Genotyping reaction was amplified on a GeneAmp PCR system 2700 (95°C for 10min, followed by 40 cycles of 95°C 15s, and 60°C 1min) and fluorescence was detected on an ABI Prism 7000 sequence detector (Applied Biosystems). Genotyping success rate was 100%, and re-screening of 7% of subjects gave 100% identical results. TaqMan Allelic Discrimination Assays was also used to genotype 8 SNPs of *HNF4A* (14), of which rs4801424 G/C (C risk allele), rs2425637 G/T (G risk allele), rs2071197 G/A (G risk allele), and rs3818247 G/T (T risk allele) have been associated with the conversion to type 2 diabetes (14). Screening methods for polymorphisms of *PGC-1A* (rs8192678 G/A - Gly482Ser; 482Ser risk allele) and *PPARG2* (rs1801282 C/G - Pro12Ala; Pro12Pro risk genotype) have been previously described (15).

Statistical analysis. The SPSS program (SPSS Inc, Chicago, Illinois, USA) version 11.0 for Windows was used in data analysis. Results are expressed as either means ± SD or percentages. Not normally distributed parameters were logarithmically transformed. Categorical variables were compared using the χ^2 and Fisher's exact tests under the dominant and recessive models, and continuous variables with the two-tailed Student's *t*-test, ANOVA or nonparametric tests, when appropriate. Logistic regression analyses and further adjustment for appropriate covariates was applied to evaluate if the SNPs predicted the development of type 2 diabetes. *P*-value of less than 0.05 was considered as statistically significant. Haploview program (16), available at http://www.broad.mit.edu/mpg/haploview/, was used to calculate and visualize the LD statistics and haplotype blocks among the SNPs. Haplotype estimation from unrelated individuals was performed by using the SNPHAP, available at http://www-gene.cimr.cam.ac.uk/clayton/software/. Permutation analysis was carried with the R 2.3.0 (17) and Design package 2.0-12 (http://biostat.mc.vanderbilt.edu/s/Design.html).

Permutation test was applied to all results giving statistically significant results. The variable 'conversion to diabetes' was permuted, and analyses were repeated 1000 times, each time re-sampling the actual dataset. *P* values of the permutation test (the number by which the permuted test statistics exceeded the nominal P values divided by the number of permutations performed) for each test of interest varied from <0.001 to 0.048, suggesting that all nominal *P*-values reported are likely to be statistically significant.

RESULTS

Eleven SNPs covering the genomic region of *PPARA* are shown in Figure 1A, and LD statistics (r^2) among the SNPs with MAF of the SNPs in Figure 1B. The distributions of the genotypes of all SNPs were in Hardy-Weinberg equilibrium and did not differ between the placebo and acarbose groups or between men and women.

Table 1 shows the conversion to diabetes (%) for all 11 SNPs under the dominant model. Analyses under the recessive model gave significant results for rs4253778 only (Table 1). A significant interaction was observed between rs1800206, rs4253776 and the treatment group in their effects on the conversion to diabetes. Therefore, the results are presented separately for the placebo and acarbose groups. In the placebo group, carriers of the rare G (162V) allele of rs1800206 converted to diabetes more often than carriers of the common CC genotype (*P* = 0.044). In the acarbose group, the conversion to diabetes was higher in carriers of the rare G allele of rs4253776 (*P* = 0.044). Moreover, a higher conversion rate (*P* = 0.030) was observed in subjects with the CC genotype of rs4253778 than in subjects having the G allele in the acarbose group (Table 1, recessive model).

The presence of the G (162V) allele of rs1800206 increased the risk of developing diabetes in the placebo group (Odds ratio (OR), 1.93, 95% CI 1.05-3.58; *P* = 0.035) (Table 2). Adjustment for confounding factors including country of origin in the multivariate logistic regression model did not abolish the statistical significance (*P* = 0.032). In the acarbose group, a 1.7-fold increase in the risk for the development of diabetes was observed in carriers of the minor G allele of rs4253776 compared to carriers of the AA genotype (dominant model), and a 2.7-fold increase in the risk in carriers of the CC genotype of rs4253778 compared to carriers of the common G allele (recessive model) (Table 2). In addition, in the placebo group, haplotypes C-G-C and A-G-C, based on SNPs rs135539, rs1800206 and rs4253778 (as reported in (10), but no other haplotypes based on different three

SNPs combinations), increased the risk to diabetes by 4.58-fold ($P = 0.010$) and 3.18-fold ($P = 0.020$), respectively, as compared to that of the C-C-C haplotype (Table 2).

Subjects in the placebo group possessing the minor G (162V) allele of rs1800206 had higher follow-up values of fasting (6.6 vs. 6.2 mmol/l, $P = 0.020$) and 2-hour (10.7 vs. 9.6 mmol/l, $P = 0.012$) glucose, higher baseline values of fasting insulin (113.7 vs. 94.3 pmol/l, $P = 0.012$), and a larger increase in 2-hour glucose levels during the follow-up (1.14 vs. 0.38 mmol/l, $P = 0.029$). Similarly, in the acarbose group, the carriers of the risk G allele of rs4253776 had significantly higher fasting and 2-hours plasma glucose levels and a larger increase in glucose levels and body weight during the follow-up, whereas carriers of the risk CC genotype of rs4253778 gained more weight and had a larger increase in waist circumference during the follow-up (data not shown).

We also tested if rs1800206 (L162V) of *PPARA* had an effect on the risk of diabetes associated previously with polymorphisms of *PGC-1A* (Gly482Ser), *PPARG2* (Pro12Ala) (15), and *HNF4A* (8 SNPs) (14). Genotypes of rs1800206 were therefore combined with those of other SNPs to form three new genotype combinations having none (0), one of two (1) and both (2) risk alleles of rs1800206 and SNPs of either *PGC-1A*, *PPARG2*, or *HNF4A*. No statistically significant interactions between the SNPs were observed with respect to diabetes risk. In the placebo group, the risk for the conversion to type 2 diabetes increased by 2.5, 2.2 and 3.4-fold with the presence of the G (162V) allele of rs1800206 and the risk alleles of either *PGC-1A* (482Ser), *PPARG2* (Pro12Pro), or *HNF4A* (G allele of rs2425637), respectively, as compared to subjects without risk alleles (Table 3).

DISCUSSION

This is the first large prospective study to report that SNPs of *PPARA* predict the development of type 2 diabetes in subjects with IGT. The minor G (162V) allele of rs1800206 was associated with a 1.9-fold ($P = 0.035$) higher risk of diabetes in the placebo group (in 6 of 7 different populations included in the study). The same allele of rs1800206 further increased the risk of diabetes beyond that associated previously with the SNPs of *PGC-1A*, *PPARG2* (15) and *HNF4A* (14). In addition, acarbose was more effective in preventing the progression from IGT to diabetes in subjects carrying either one or both risk alleles of *PPARA* and *PGC-1A* or *HNF4A*, indicating a gene-treatment interaction.

Activation of PPAR-α favours lipid sparing (1) and improves insulin sensitivity and β-cell function by decreasing adiposity, skeletal muscle and hepatic steatosis, and plasma free fatty acids and triglycerides (3-5). Moreover, recent studies show that PPAR-α deficiency decreases the mean area of the pancreatic β-cells and diminishes insulin secretion in response to glucose (18), whereas PPAR-α agonists reduce lipotoxicity in human islets and prevent the progression to diabetes (18,19). It is therefore possible that the pancreatic effects of PPAR-α are responsible for the results observed in our study.

rs1800206 of *PPARA* is likely to be functional. Transcriptional studies have demonstrated that the C to G change in codon 162 (L162V) of exon 5 encodes a more active PPAR-α *in vitro* (8,20). However, the L162V polymorphism has different effects on gene transcription depending on the concentration of ligand. In the presence of high concentrations, transcriptional activity of the G (162V) allele is higher compared to that of the C allele (8,20), whereas at low concentrations results are opposite (20). In our and previous studies (10) the presence of the G (162V) allele increased the risk of diabetes. The reasons for this finding remain unexplained but it is possible that the effect of rs1800206 is influenced by gene-

environment interactions regulating the concentrations of ligands for PPAR-α. Furthermore, the L162V substitution could be in LD with another SNP causing the association.

What could be the mechanisms how acarbose and SNPs in *PPARA* as well as in *PGC-1A*, *PPARG2* and *HNF4A* regulate the risk of diabetes? High levels of free fatty acids and postprandial hyperglycemia (21) increase the production of reactive oxygen species (ROS) and insulin resistance. PPAR-α protects from oxidative stress, and PGC-1α prevents the formation of ROS by transcriptionally regulating the mitochondrial antioxidant defence system (22). Since acarbose alleviates postprandial hyperglycemia and lowers the levels of free fatty acids, it could interact with SNPs of *PPARA* and *PGC-1A* and thus prevent the development of diabetes.

The Gly482Ser substitution of *PGC-1A* (15,23), the Pro12Ala substitution of *PPARG2* (15,24) and SNPs of *HNF4A* (14) have been associated previously with the risk of diabetes. PGC-1α co-activates PPAR-α in the transcriptional control of fatty acid oxidation proteins (2), whereas hepatic expression of PPAR-α is regulated by HNF4-α (25). Genetically determined defects in one or several factors of this transcriptional network might impair their function, and thus affect the entire system. In the placebo group, the simultaneous presence of the minor G allele (162V) of rs1800206 of *PPARA* with the 482Ser allele of *PGC-1A* (rs8192678), the Pro12Pro genotype of *PPARG2* (rs1801282) or the G allele of *HNF4A* (rs2425637) increased the risk of diabetes by 2.5-, 2.2- and 3.4-fold, respectively. However, no statistically significant interaction between the SNPs with respect to the conversion to diabetes were observed. Mechanisms explaining possible transcriptional interactions on the risk of diabetes are currently unclear.

Examination of many genetic variations and their interactions on several variables raises the possibility of finding false positive results. Bonferroni correction is conservative and likely to result in over-adjustment. All statistically significant results survived the permutation test which suggests that it is unlikely that our findings have occurred by chance.

In conclusion, the present study shows that SNPs of *PPARA* modulate the progression to type 2 diabetes in subjects with IGT. Moreover, the combination of rs1800206 of *PPARA* with SNPs of *PGC-1A*, *PPARG2* and *HNF4A* had an additive effect on the risk of diabetes.

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TABLE 1

Conversion to type 2 diabetes (%) according to SNPs of *PPARA* under the dominant model

	rs4253623 A/G		**rs135547 C/G**		**rs135542 T/C**		**rs135539 A/C**		**rs4253701 G/A**		**rs8138102 A/G**	
	AA	AG+GG	CC	CG+GG	TT	TC+CC	AA	AC+CC	GG	GA+AA	AA	AG+GG
n = 767	585	167+15	368	323+76	404	306+57	255	373+139	610	153+4	454	269+44
(% of total *n*)	76.3	23.7	48.0	52.0	52.7	47.3	33.3	66.7	79.5	20.5	59.2	40.8
All subjects	42.7	33.0‡	38.3	42.4	38.9	42.1	42.5	39.5	40.2	41.4	39.6	41.5
Placebo	44.8	38.8	43.1	48.4	43.3	48.6	49.6	44.6	45.4	48.7	45.5	46.7
Acarbose	36.4	25.3	33.3	34.5	34.5	33.1	35.7	32.9	33.9	34.2	32.9	35.4

	rs4253728 G/A		**rs1800206 C/G***		**rs11090819 G/A**		**rs4253776 A/G***		**rs4253778 G/C**		**rs4253778 G/C†**	
	GG	GA+AA	CC	CG+GG	GG	GA+AA	AA	AG+GG	GG	GC+CC	GG+GC	CC
n = 767	414	286+67	670	93+4	654	106+7	591	160+16	497	230+40	497+230	40
(% of total *n*)	54.0	46.0	87.4	12.6	85.3	14.7	77.1	22.9	64.8	35.2	94.8	5.2
All subjects	40.2	40.8	39.9	44.3	39.8	44.2	39.8	42.6	39.8	41.5	39.6	55.0
Placebo	45.7	46.6	44.1	60.4§	44.3	57.4	47.3	41.7	45.4	47.5	45.7	52.6
Acarbose	33.7	34.1	34.8	28.6	34.2	32.2	31.0	43.8‖	33.3	34.9	32.4	57.1¶

**P* value for the interaction with treatment < 0.05. †Recessive model for combination of genotype groups. ‡*P* = 0.020 (0.019). §*P* = 0.044 (0.036). ‖*P* = 0.044 (0.030). ¶*P* = 0.030 (0.033): *P* value compares the conversion to diabetes between the two genotype groups; Fisher's exact test. *P* value after the permutation testing in parentheses.

TABLE 2

SNPs of *PPARA* as predictors for the development of type 2 diabetes by the treatment group (logistic regression analysis, dominant and recessive models; haplotype analysis)

	Placebo group			Acarbose group			
Single-SNP analysis	OR (95% CI)	*P*	*P**	OR (95% CI)	*P*	*P**	*P*†
Model 1‡							
rs1800206 C/G	**1.93** (1.05-3.58)	**0.035**	**0.036**	0.75 (0.39-1.46)	0.397		**0.040**
	2.02 (1.06-3.84)	**0.032**‖	**0.021**‖				
Model 2‡							
rs4253776 A/G	0.80 (0.50-1.26)	0.331		**1.73** (1.04-2.88)	**0.035**	**0.030**	**0.027**
				1.70 (1.00-2.90)	0.050‖	0.091‖	
Model 3§							
rs4253778 G/C	1.32 (0.52-3.32)	0.554		**2.78** (1.14-6.79)	**0.025**	**0.008**	0.335
				2.28 (0.88-5.86)	0.089‖		
Haplotype analysis: rs135539 A/C-rs1800206 C/G-rs4253778 G/C							
C-C-G (*n* = 357)	1.66 (0.93-2.98)	0.089		0.94 (0.47-1.89)	0.942		0.512
C-G-C (*n* = 39)	**4.58** (1.43-14.67)	**0.010**	**0.007**	0.40 (0.12-1.40)	0.152		
	3.84 (1.14-12.92)	**0.030**‖	**0.038**‖				
A-G-C (*n* = 45)	**3.18** (1.20-8.43)	**0.020**	**0.018**	1.36 (0.47-3.92)	0.570		
	3.27 (1.19-9.01)	**0.022**‖	**0.035**‖				

**P* value after the permutation testing. †*P* value for the interaction with treatment. ‡Dominant model. §Recessive model. ‖Adjusted for age, gender, smoking, weight at baseline, weight change and country of origin. 95% CI = 95% confidence interval; OR = Odds ratio. Smoking was coded as 0 = never smokers and ex-smokers and 1 = current smokers at baseline. rs1800206 C/G genotypes were coded as 0 = CC, 1 = CG+GG. rs4253776 A/G genotypes were coded as 0 = AA, 1 = AG+GG. rs4253778 G/C genotypes were coded as 0 = GG+GC, 1 = CC. In haplotype analysis (rs135539 A/C-rs1800206 C/G-rs4253778 G/C), coding was as follows: 0 = C-C-C (*n* = 109), 1 = C-C-G (*n* = 357), 2 = C-G-C (*n* = 39), 3 = A-G-C (*n* = 45).

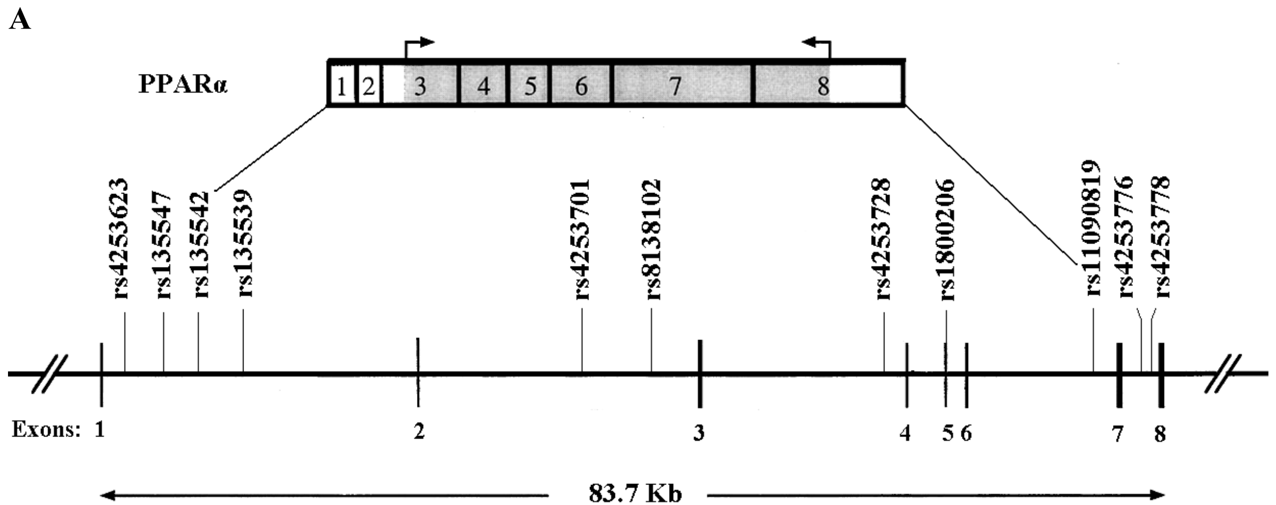
TABLE 3
SNP rs1800206 C/**G** of *PPARA* in combination the Gly482Ser polymorphism (rs8192678 G/**A**) of *PGC-1A* (Model 1), the Pro12Ala polymorphism (rs1801282 **C**/G) of *PPARG2* (Model 2), and the SNP rs2425637 **G**/T of *HNF4A* (Model 3) as predictors for the development of type 2 diabetes by treatment group (logistic regression analysis)

		Placebo group			Acarbose group		
		OR (95% CI)	*P*	*P**	OR (95% CI)	*P*	*P*†
Model 1							
PPARA rs1800206 *PGC-1A* rs8192678	One risk allele (1)	**1.77** (1.17-2.68)	**0.007**	**0.009**	0.75 (0.47-1.20)	0.227	**0.001**
		1.78 (1.16-2.73)	**0.009**§	**0.012**§			
	Both risk alleles (2)	**2.57** (1.08-6.08)	**0.032**	**0.019**	0.53 (0.22-1.27)	0.154	
		2.79 (1.13-6.87)	**0.026**§	**0.041**§			
Model 2							
PPARA rs1800206 *PPARG2*rs1801282	One risk allele (1)	1.00 (0.61-1.64)	0.988		1.51 (0.86-2.66)	0.156	0.531
	Both risk alleles (2)	**2.27** (1.03-4.97)	**0.040**	**0.038**	1.12 (0.48-2.61)	0.788	
		2.39 (1.05-5.43)	**0.038**§	**0.035**§			
Model 3							
PPARA rs1800206 *HNF4A* rs2425637	One risk allele (1)	**1.72** (1.02-2.91)	**0.042**	**0.048**	0.64 (0.36-1.14)	0.129	**0.001**
		1.99 (1.14-3.48)	**0.016**§	**0.012**§			
	Both risk alleles (2)	**3.40** (1.54-7.51)	**0.002**	**<0.001**	0.54 (0.23-1.29)	0.164	
		3.99 (1.73-9.18)	**0.001**§	**0.001**§			

P* value after the permutation testing. †*P* value for the interaction with treatment. §Adjusted for age, gender, smoking, weight at baseline, weight change and country of origin. Model 1: rs1800206 C/G** + rs8192687 G/**A** genotypes were coded as: (0)=CC+GG; (1)=CC+(GA+AA) and (CG+GG)+GG; (2)=(CG+GG)+(GA+AA). Model 2: rs1800206 C/**G** + rs1801282 **C**/G genotypes were coded as: (0)=CC+(CG+GG); (1)=CC+CC and (CG+GG)+(CG+GG); (2)=(CG+GG)+CC. Model 3: rs1800206 C/**G** + rs2425637 **G**/T genotypes were coded as: (0)=CC+TT; (1)=CC+(GG+GT) and (CG+GG)+TT; (2)=(CG+GG)+(GG+GT).

FIG. 1. Schematic map of 83.7-Kb genomic region showing the approximate location of 11 SNPs genotyped in *PPARA*. Upper panel represents the *PPARA* messenger RNA and arrows indicate the start and finish sites of translation (modified from Vohl MC *et al.*, 2000) (A). Linkage disequilibrium statistics (r^2) among the SNPs of *PPARA* and the minor allele frequency (MAF) (B). SNPs are coded by National Centre for Biotechnology Information's dbSNP accession numbers

A



B

	rs4253623										
rs135547	0.062	**rs135547**									
rs135542	0.056	0.829	**rs135542**								
rs135539	0.195	0.439	0.372	**rs135539**							
rs4253701	0.005	0.015	0.003	0.002	**rs4253701**						
rs8138102	0.016	0.006	0.010	0.053	0.297	**rs8138102**					
rs4253728	0,031	0.014	0.017	0.085	0.050	0.443	**rs4253728**				
rs1800206	0.010	0.004	0.005	0.028	0.190	0.079	0.187	**rs1800206**			
rs11090819	0.013	0.004	0.008	0.023	0.031	0.009	0.049	0.421	**rs11090819**		
rs4253776	0.011	0.005	0.010	0.023	0.000	0.370	0.294	0.001	0.005	**rs4253776**	
rs4253778	0.024	0.001	0.002	0.050	0.015	0.310	0.358	0.164	0.335	0.565	**rs4253778**
MAF	0.13	0.31	0.27	0.43	0.11	0.23	0.27	0.07	0.08	0.13	0.20