Disease Markers 29 (2010) 259–264
DOI 10.3233/DMA-2010-0756
IOS Press

Homozygosity for the Ala allele of the PPARγ2 Pro12Ala polymorphism is associated with reduced risk of coronary artery disease

Andrea Galgani[a,b,*], AnaMaria Valdes[c], Henry A. Erlich[d], Calvin Mano[d], Suzanne Cheng[d], Antonio Petrone[a], Federica Sentinelli[e], Andrea Berni[f], Marco G. Baroni[e] and Raffaella Buzzetti[a]
[a]*Department of Clinical Sciences, University of Rome "La Sapienza", Rome, Italy*
[b]*Centro di Servizi Interdipartimentale, Stazione per la Tecnologia Animale, University of "Tor Vergata", Rome, Italy*
[c]*Twin Research Unit, King's College London, UK*
[d]*Human Genetics Department, Roche Molecular System, Inc., Pleasanton, CA, USA*
[e]*Department of Medical Sciences, Endocrinology and Metabolism, University of Cagliari, Cagliari, Italy*
[f]*Department of Cardiology, II Faculty of Medicine, University of Rome "La Sapienza", Rome, Italy*

Abstract. Several studies suggest that the peroxisome proliferator-activated receptor gamma (PPARγ) is involved in atherogenesis. The Pro12Ala polymorphism in the gene encoding PPARγ (*PPAR*γ2gene) influences the risk for type 2 diabetes. Two population-based studies have shown that the Ala allele is associated with reduced carotid intimal-medial thickness (IMT). However, studies focusing on acute clinical events have yielded conflicting results. Our aim was to evaluate the role of the Pro12Ala *PPAR*γ2 polymorphism on the risk of coronary artery disease (CAD) in an Italian population with a case-controlled genetic association study in which 478 CAD patients and 218 controls were genotyped for the Pro12Ala polymorphism. CAD was diagnosed by angiography. We found that homozygotes for the Ala12 allele had a significantly reduced risk of CAD after adjusting for diabetes, sex, age, body mass index (BMI), smoking, lipids and hypertension (OR $= 0.007$; 95% C.I. $= 0.00$–0.32 $p < 0.011$). In this case-control study, homozygosity for the Ala allele at codon 12 of the *PPAR*γ2 gene resulted in reduced risk of CAD. This is consistent with reports from previous studies focusing on atherosclerosis and myocardial infarction.

Keywords: Coronary artery disease (CAD), Peroxisome Proliferator-Activated Receptor Gamma 2 (PPARγ2), Myocardial infarction (MI), Pro12Ala polymorphism

1. Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor expressed in endothelial cells, vascular smooth muscle cells, monocytes/macrophages, and T-cells [1]. It is thought to be the master regulator of fat storage. Experimental work suggests that PPARγ also acts directly on local vasculature during several critical phases of atherothrombosis, which suggests that it may be an important determinant of gene expression during atherogenesis [2]. The role of PPARγ in atherogenesis has been widely investigated with the aid of glitazones and other PPARγ activators [1]. These agents have been

*Corresponding author: Andrea Galgani PhD, Centro di Servizi Interdipartimentale, Stazione per la Tecnologia Animale, University of "Tor Vergata", Via Montpellier 1, Rome, Italy. Tel.: +39 06 72596396; Fax: +39 06 72596037; E-mail: galgani@scienze.uniroma2.it.

ISSN 0278-0240/10/$27.50 © 2010 – IOS Press and the authors. All rights reserved

shown to inhibit the growth, proliferation [4], and migration of vascular smooth muscle cell, and they also appear to exert anti-inflammatory and potentially anti-atherogenic effects on monocytes and macrophages [5, 6]. PPARγ also regulates macrophage lipid homeostasis [6]. In clinical trials, treatment with PPARγ activators was found to attenuate intimal and medial complex thickening [7,8] and narrowing of the coronary lumen [9]. Collectively, these findings provide a strong biological rationale for the hypothesis that susceptibility to atherosclerosis and cardiovascular disease can be influenced by variations in the *PPARγ2* gene.

The Pro12Ala polymorphism has in fact been linked to a reduced risk of type 2 diabetes (T2D) [10] and to enhanced insulin sensitivity [11]. The alanine substitution at residue 12 decreases PPARγ2's binding affinity for the cognate promoter element, thereby diminishing its ability to transactivate responsive promoters [12]. This leads to increased leptin expression with upregulated fatty acid combustion and decreased lipogenesis, which ultimately reduce the cell's triglyceride content [13].

Two population-based studies have previously explored the influence of the *PPARγ2* Pro12Ala polymorphism on atherosclerosis by quantifying carotid intima-media thickness (IMT). In a study of the Canadian Oji-Cree aboriginal peoples, the presence of the Ala12 allele was associated with reduced IMT [14]. A German study on subjects at risk for diabetes obtained similar results [15] although they reported that only homozygotes for the Ala12 allele had significantly reduced IMT.

In a large longitudinal study, Ridker and co-workers [16] found that Ala12 carriers were protected against myocardial infarction (MI), consistent with the decreased IMT results mentioned above. However, two studies which focused on different cardiovascular clinical end-points have reported results at variance to these findings. The study by Blüher and co-workers [17] found that diabetic carriers of the Ala12 allele were not at reduced coronary heart disease (CHD) risk, as established by a history of MI, coronary bypass operations or significant stenosis of at least one coronary artery. More recently, Pischon and co-workers [18] found that Ala12 carriers were not at reduced risk of fatal CHD or nonfatal MI, but that, among overweight subjects, Ala12 carriers were actually at increased risk. This indicated the possibility of high BMI and presence of T2D constituting risk factors sufficiently strong for overcoming the risk effect of the Pro12 Ala polymorphism on CHD.

Association of the *PPARγ2* Pro12Ala polymorphism with T2D disease risk has been consistently replicated [10], building a strong argument for involvement of this variation with this disease [19]. Association of the polymorphism with coronary events has not been so consistently replicated, perhaps due to disease complexity and heterogeneity among cases. In this study we focused upon sub-clinical disease, comparing patients with angiographic evidence for coronary atherosclerosis to healthy controls to evaluate the risk of atherosclerosis with *PPARγ2* Pro12Ala genotypes.

2. Methods

The protocol for this case-control study was reviewed and preapproved by the Ethics Committee of University of Rome, Italy. Written informed consent to all procedures and to publication of the results of the study was obtained from all participants. Unless otherwise specified, all commercial products mentioned below were used according to manufacturers' instructions.

2.1. Study cohorts

A total number of 696 Caucasian subject were consecutively recruited in the Centre-West Coast of Italy, most from Rome and its surrounding towns. The case cohort consisted of 478 patients (380 men and 98 women, aged 36 to 83 years) with coronary artery disease (CAD) defined as documented evidence of a previous myocardial infarction (MI) – see below – or coronary catheterization findings of stenosis of 50% or more in at least one major coronary artery together with clinical symptoms of angina. Myocardial infarction was defined as the presence of typical electrocardiographic changes and elevation in the levels of at least two enzymes and confirmed by the presence of wall motion abnormalities on left ventriculography. The control cohort consisted of 218 healthy unrelated individuals (48 men and 170 women, aged 35 to 70 years) with neither CAD (reflected by responses to the Rose questionnaire and ECG findings [Minnesota coding]), nor T2D (no current or past use of oral hypoglycemic drugs/insulin) plus a normal fasting blood glucose level (i.e., $>$ 126 mg/dl or 7.0 mmol/L).

Using a standard questionnaire, researchers interviewed each participant to obtain a complete medical history, including detailed information on smoking habits, comorbidities, and current medications. Body mass index (BMI), blood pressure, fasting glucose, to-

Table 1
Clinical characteristics of study participants

	Controls	CAD all	CAD no T2D	CAD T2D
	$N = 218$	$N = 478$	$N = 338$	$N = 140$
Age (years)*	49.1 ± 7.8	62.1 ± 9.1	61.5 ± 8.9	63.6 ± 9.1
Sex (F/M)*	170/48	97/381	67/271	30/110
BMI (kg/m^2)*	24.1 ± 3.7	27.3 ± 3.4	27.1 ± 3.2	27.9 ± 4.0
Diabetes n (%)*	0	141 (29.4)	0	140 (100.0)
Hypertension n (%)*	23 (10.6)	282 (59.0)	189 (55.8)	93 (66.4)
Dyslipidemia n (%)*	52 (23.9)	199 (41.6)	144 (42.7)	54 (38.6)
Angina n (%)*	0	88 (18.4)	55 (16.3)	33 (23.6)
Non fatal MI n (%)*	0	210 (43.9)	140 (41.5)	70 (50.0)
Smokers n (%)*	64 (29.4)	113 (23.7)	83 (24.5)	30 (21.7)
Triglycerides (mg/dL)*	98.0 ± 54.7	130.4 ± 74.2	125.3 ± 67.8	142.7 ± 86.7
Glucose (mg/dL)*	91.3 ± 10.5	112.4 ± 48.3	96.7 ± 22.5	150.1 ± 68.7
Total cholesterol (mg/dL)*	204.0 ± 32.9	172.3 ± 47.3	171.3 ± 45.6	174.0 ± 51.2
HDL cholesterol (mg/dL)*	56.8 ± 15.2	41.9 ± 13.9	42.3 ± 14.2	40.8 ± 13.0
*PPARγ2***Pro12Ala:**				
Ala allele frequency %	8.2% ($n = 18$)	7.7% ($n = 37$)	7.3% ($n = 25$)	8.9% ($n = 12$)
Ala-Ala frequency%	1.8%	0.2%	0.0%	0.7%

Abbreviations – M indicates male; F indicates female; CAD = Coronary Artery Disease; T2D = Type 2 Diabetes; BMI = Body Mass Index; MI = Myocardial Infarction. Age, BMI, triglycerides, glucose, total cholesterol and HDL cholesterol are given as mean ±SD. Other values represent number of individuals (n) with percentage (n/N) in parentheses.
*$P < 0.001$ between all CAD patients ($n = 478$) and controls for all continuous and categorical variables with the exception for HDL cholesterol ($P < 0.04$) and total cholesterol and smokers (not significant). The statistical analysis for age, BMI, triglycerides, glucose, total cholesterol and HDL cholesterol was performed on log-transformed values. Continuous variables have been compared by t-test and categorical variables by χ^2 test. Significance was evaluated by 2-tailed distribution of P value.

tal and HDL cholesterol, and triglyceride levels were measured in all subjects at baseline. Participants were classified as having hypertension if they presented elevated systolic ($>$ 140 mmHg) and/or diastolic ($>$ 90 mmHg) blood pressure and/or were currently using anti-hypertensive medication. Dyslipidemia was defined as a total plasma cholesterol level $\geqslant$ 200 mg/dL and/or current use of cholesterol-lowering medication.

2.1.1. Laboratory methods

Plasma levels of cholesterol and triglycerides were measured with a Technicon RA-1000 Autoanalyzer. HDL cholesterol was measured after precipitation of apoB-containing lipoproteins with photungstic acid-$MgCl_2$, and the LDL cholesterol level was estimated with the Friedewald formula [11]. Blood glucose levels were measured with the glucose oxidase method (Autoanalyzer, Beckman Coulter, Inc., Fullerton, CA, USA).

2.1.2. Genotyping

*PPARγ*2 Pro12Ala genotypes were generated with a multi-locus, PCR-based assay with immobilized, sequence-specific oligonucleotide probes, as described elsewhere [20] with minor modifications. Genotyping was performed under blinded conditions, and the results were reviewed with in-house image analysis software developed by Roche Molecular Systems.

2.1.3. Statistical methods

A logistic regression analysis was conducted with CAD as the outcome variable and the *PPAR* γ 2 Pro12Ala genotype as the independent variable. The latter was coded 0/1 for the recessive model (0 = Pro-Pro, Pro-Ala, 1 = Ala-Ala) or 0/1/2 for the additive model (depending on the number of Ala allele copies found). Age, sex, fasting glucose, triglycerides, total and HDL cholesterol levels, BMI, hypertension, and smoking habitus were included as covariates. Controls were compared with the total case cohort as well as with subgroups of the latter defined by MI or T2D status. All analyses were conducted with S-Plus 6.1 software (Insightful Corp.) Differences between the genotype distributions in cases and controls were compared with those expected under Hardy Weinberg Equilibrium. Differences were assessed with the Pearson chi-squared test, and no significant deviations were observed.

3. Results

The clinical characteristics according to disease status are shown in Table 1. The case cohort displayed a

Table 2
Calculated odds ratios for CAD for the *PPAR*γ2 genotype in different patient subgroups

CAD		Additive model			Recessive model		
subgroup	n CAD cases	O.R.	95% CI	p-value	O.R.	95% CI	p-value
all	478	0.931	0.61–1.42	0.740	0.007	0.00–0.32	0.011
no MI	268	0.718	0.29–1.80	0.480	0.015	0.00–0.58	0.024
MI	210	1.047	0.62–1.78	0.865	0.015	0.00–643.1	0.439
no T2D	338	0.839	0.37–1.92	0.677	0.014	0.00–15.97	0.235
T2D	140	0.775	0.36–1.66	0.511	0.063	0.00–2.25	0.130

Abbreviations – CAD = Coronary Artery Disease; O.R. = Odds Ratio; CI = Confidence Interval; MI = Myocardial Infarction; T2D = Type 2 Diabetes;
Subgroup – no MI = CAD+/MI-; MI = CAD+/MI+; no T2D = CAD+/T2D-; T2D = CAD+/T2D+;
Age, sex, fasting glucose, triglycerides, total and HDL cholesterol, BMI, hypertension and smoking status were included as covariates in the logistic regression analysis.

lower proportion of females and a significantly higher mean age than the control cohort, but all analyses were adjusted for these and other potentially confounding factors. The allele and genotype frequencies among controls were comparable to those reported among Caucasians in other studies [14].

As shown in Table 2, A1a12 heterozygosity had no significant effect on the risk of CAD, but Ala-Ala homozygotes had significantly lower risks than Pro12 carriers. This difference was also significant when controls were compared with the subgroup of CAD cases with no history of MI. No significant association between the *PPAR*γ2 genotype and CAD was observed when patients were divided into T2D and non-T2D groups, although the value of the odds ratio was similar to that obtained for the entire set for both T2D-affected and T2D-unaffected subsets. When MI and T2D status were included in the model, an OR of 0.015 (95% CI = 0.00–0.81, $p < 0.04$) was obtained, indicating that its protective effect is not dependent on the T2D or MI status of the CAD cases.

4. Discussion

Our results demonstrate that *PPAR*γ2Ala12 homozygotes have a significantly lower risk for CAD, independent of T2D status. These findings are consistent with previous reports indicating that the Ala12 allele protects against MI [16] and atherosclerosis [14,15]. As reported by Temelkova-Kuktshiev et al. [15], the decreased risk of atherosclerosis was observed only in Ala12 homozygotes, not among Pro-Ala heterozygotes. Two previous studies failed to identify any association between *PPAR*γ2Ala12 and atherosclerosis [17, 18], but both the cases and controls in these studies had significant risk factors (either T2D or obesity) that may have rendered the risk associated with the Pro12 Ala polymorphism less discernable.

Although *PPAR*γ2 Pro12Ala has been widely reported to affect risk of T2D [10–12], *PPAR*γ2 allele and genotype frequencies were not significantly different in T2D+ CAD patients vs. controls or in CAD patients with vs. without T2D. In fact, the highest Ala allele frequency was – surprisingly – found in the subset of CAD patients who also had T2D (8.9% compared to the 8.2% in the controls and 7.3% in non-diabetic CAD patients). Although this is counterintuitive, we note that the T2D CAD subgroup was also the smallest and that the limited sample size may explain this finding. In addition, the current sample sizes were not large enough to detect modest differences in allele frequencies ($< 2\%$ in the present study) as statistically significant, and an odds ratio of 2.0 (or 0.5 depending on the direction of the association) is required to achieve $p < 0.05$ for 80%. The protective effect of Ala12 on CAD reported here does not seem to be mediated by the well-established association of Ala12 with T2D.

There are some limitations to the present study. First, the cases and controls were not matched for age and sex. However, these two factors are unlikely to influence distribution of the *PPAR*γ2 genotype and therefore have been included as covariates in the logistic regression analysis. In addition, the number of Ala-Ala homozygotes in this study was not large. Nonetheless, the results reported here are consistent with those found in two previous studies of IMT (14–15), suggesting that our conclusions are unlikely to be the result of a spurious protective association.

The data regarding the genetic association of this polymorphism with cardiovascular phenotypes, however, appear to conflict with some previously published clinical data using PPARγ activator compounds. The *PPAR*γ2 alanine allele encodes a protein with reduced

activity [12], yet clinical data indicate that activators of PPARγ, like thiazolidinediones (TZD), reduce IMT and other CHD risk factors.

A similar contradiction is seen with regard to lipid metabolism and insulin sensitivity:

Yamauchi et al. [13] reported that supraphysiological TZD-activation of PPARγ increased the triglyceride content of white adipose tissue and decreased that of the liver and muscle tissue. The result was increased insulin sensitivity. In contrast, moderate reduction of PPARγ activity decreased the triglyceride content of white adipose tissue, skeletal muscle, and liver tissue by upregulating leptin expression. In this case, too, insulin sensitivity was enhanced by leptin-induced increases in fatty-acid combustion and decreases in lipogenesis. The authors concluded that upregulation and downregulation of PPARγ activity improve insulin resistance, albeit by different mechanisms. A similar explanation might account for the paradoxically similar effects on atheroscelrosis PPARγ agonists and the *PPARγ*2 Pro12Ala variant on risk of atherosclerosis. Functional experiments are needed to address this issue.

In summary, we report a significant association with reduced risk of CAD in carriers of the Ala12Ala genotype, providing further evidence on the role of the PPARγ gene in insulin-resistance and atherosclerosis.

Acknowledgements

We thank Dott. Rudolf Meiswinkel for copyediting service. This work has been supported in part by grants received from the Italian "Ministero dell'Università e Ricerca Scientifica e Tecnologica" to R.B. (FIRB 2001 RBNE01C5SZ_004; PRIN 2001 2001061775_007; PRIN 2003 2003061834_004)

References

[1] N. Marx, H. Duez, J.C. Fruchart and B. Staels, Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells, *Circ Res* **94** (2004), 1168-1178.

[2] J. Plutzky, Peroxisome proliferator-activated receptors in vascular biology and atherosclerosis: emerging insights for evolving paradigms, *Curr Atheroscler Rep* **2** (2000), 327–335.

[3] S. Goetze, X.P. Xi, H. Kawano, T. Gotlibowski, E. Fleck, W.A. Hsueh and R.E. Law, PPAR gamma-ligands inhibit migration mediated by multiple chemoattractants in vascular smooth muscle cells, *J Cardiovasc Pharmacol* **33** (1999), 798–806.

[4] S. Wakino, U. Kintscher, S. Kim, F. Yin, W.A. Hsueh and R.E. Law, Peroxisome proliferator-activated receptor gamma ligands inhibit retinoblastoma phosphorylation and G1→S transition in vascular smooth muscle cells, *J Biol Chem* **275** (2000), 22435–22441.

[5] M. Ricote, A.C. Li, T.M. Willson, C.J. Kelly and C.K. Glass, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation, *Nature* **391** (1998), 79–82.

[6] J.S. Welch, M. Ricote, T.E. Akiyama, F.J. Gonzalez and C.K. Glass, PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages, *Proc Natl Acad Sci* **100** (2003), 6712–6717.

[7] J. Minamikawa, S. Tanaka, M. Yamauchi, D. Inoue and H. Koshiyama, Potent inhibitory effect of troglitazone on carotid arterial wall thickness in type 2 diabetes, *J Clin Endocrinol Metab* **83** (1998), 1818–1820.

[8] H. Koshiyama, D. Shimono, N. Kuwamura, J. Minamikawa and Y. Nakamura, Rapid communication: inhibitory effect of pioglitazone on carotid arterial wall thickness in type 2 diabetes, *J Clin Endocrinol Metab* **86** (2001), 3452–3456.

[9] T. Takagi, T. Akasaka, A. Yamamuro, Y. Honda, T. Hozumi, S. Morioka and K. Yoshida, Troglitazone reduces neointimal tissue proliferation after coronary stent implantation in patients with non-insulin dependent diabetes mellitus: a serial intravascular ultrasound study, *J Am Coll Cardiol* **36** (2000), 1529–1535.

[10] D. Altshuler, J.N. Hirschhorn, M. Klannemark, C.M. Lindgren, M.C. Vohl, J. Nemesh, C.R. Lane, S.F. Schaffner, S. Bolk, C. Brewer, T. Tuomi, D. Gaudet, T.J. Hudson, M. Daly, L. Groop and E.S. Lander, The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes, *Nat Genet* **26** (2000), 76–80.

[11] R. Buzzetti, A. Petrone, M.C. Ribaudo, I. Alemanno, S. Zavarella, C.A. Mein, F. Maiani, C. Tiberti, M.G. Baroni, E. Vecci, M. Arca, F. Leonetti and U. Di Mario, The common PPAR-gamma2 Pro12Ala variant is associated with greater insulin sensitivity, *Eur J Hum Genet* **12** (2004), 1050–1054.

[12] S.S. Deeb, L. Fajas, M. Nemoto, J. Pihlajamaki, L. Mykkanen, J. Kuusisto, M. Laakso, W. Fujimoto and J. Auwerx, A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity, *Nat Genet* **20** (1998), 284–287.

[13] T. Yamauchi, J. Kamon, H. Waki, K. Murakami, K. Motojima, K. Komeda, T. Ide, N. Kubota, Y. Terauchi, K. Tobe, H. Miki, A. Tsuchida, Y. Akanuma, R. Nagai, S. Kimura and T. Kadowaki, The mechanisms by which both heterozygous peroxisome proliferator-activated receptor (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance, *J Biol Chem* **276** (2001), 41245–41254.

[14] K.Z. Al-Shali, A.A. House, A.J. Hanley, H.M. Khan, S.B. Harris, B. Zinman, M. Mamakeesick, A. Fenster, J.D. Spence and R.A. Hegele, Genetic variation in PPARG encoding peroxisome proliferator-activated receptor gamma associated with carotid atherosclerosis, *Stroke* **35** (2004), 2036–2040.

[15] T. Temelkova-Kurktschiev, M. Hanefeld, G. Chinetti, C. Zawadzki, S. Haulon, A. Kubaszek, C. Koehler, W. Leonhardt, B. Staels and M. Laakso, Ala12Ala genotype of the peroxisome proliferator-activated receptor gamma2 protects against atherosclerosis, *J Clin Endocrinol Metab* **89** (2004), 4238–4242.

[16] P.M. Ridker, N.R. Cook, S. Cheng, H.A. Erlich, K. Lindpaintner, J. Plutzky and R.Y. Zee, Alanine for proline substitu-

tion in the peroxisome proliferator-activated receptor gamma-2 (PPARG2) gene and the risk of incident myocardial infarction, *Arterioscler Thromb Vasc Biol* **23** (2003), 859–863.

[17] M. Blüher, T. Klemm, T. Gerike, H. Krankenberg, G. Schuler and R. Paschke, Lack of association between peroxisome proliferator-activated receptor-gamma-2 gene variants and the occurrence of coronary heart disease in patients with diabetes mellitus, *Eur J Endocrinol* **146** (2002), 545–551.

[18] T. Pischon, J.K. Pai, J.E. Manson, F.B. Hu, K.M. Rexrode, D. Hunter and E.B. Rimm, Peroxisome proliferator-activated receptor-gamma2 P12A polymorphism and risk of coronary heart disease in US men and women, *Arterioscler Thromb Vasc Biol* **25** (2005), 1654–1658.

[19] G.P. Page, V. George, R.C. Go, P.Z. Page and D.B. Allison, Are we there yet: Deciding when one has demonstrated specific genetic causation in complex diseases and quantitative traits, *Am J Hum Genet* **73** (2003), 711–719.

[20] S. Cheng, M.A. Grow, C. Pallaud, W. Klitz, H.A. Erlich, S. Visvikis, J.J. Chen, C.R. Pullinger, M.J. Malloy, G. Siest and J.P. Kane, A multilocus genotyping assay for candidate markers of cardiovascular disease risk, *Genome Res* **9** (1999), 936–949.