# Impact of Two Common Polymorphisms in the *PPARγ* Gene on Glucose Tolerance and Plasma Insulin Profiles in Monozygotic and Dizygotic Twins

## Thrifty Genotype, Thrifty Phenotype, or Both?

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**The Pro12Ala polymorphism in the *PPARγ2* gene has been associated with reduced risk of type 2 diabetes and insulin resistance. Recently, an association between dizygotic twinning and *PPARγ* gene polymorphisms has been proposed. We investigated the phenotypic appearance of the two polymorphisms (Pro12Ala and exon 6 C→T) in *PPARγ* among elderly twins (207 monozygotic [MZ] and 342 dizygotic [DZ]) and evaluated whether they could explain previously reported differences in plasma glucose and insulin profiles among MZ and DZ twins. We demonstrated a significant impact of the Pro12Ala polymorphism on glucose tolerance, diabetic status, homeostasis model assessment for insulin resistance, and plasma insulin profiles in twins. No impact of the silent exon 6 polymorphism on glucose homeostasis or plasma insulin profiles was found. Independent of the polymorphisms, we observed a significant impact of zygosity status per se on the plasma insulin profile after oral glucose ingestion, with the MZ twins being more hyperinsulinemic, indicating insulin resistance, than the DZ twins. Nonsignificantly higher glucose concentrations were observed among MZ compared with DZ twins. We demonstrated an association between the Ala allele and reduced risk of diabetes and insulin resistance in twins. However, the differences in metabolic profiles among MZ and DZ twins were not explained by differences in frequencies of the genetic variants and may be due to intrauterine environmental factors operating in twins independent of genotype. Accordingly, our study simultaneously supports a role for both the intrauterine environment (thrifty phenotype) and for genetics (thrifty genotype) in the etiology of insulin resistance and perhaps glucose intolerance in twins. *Diabetes* 52: 194–198, 2003**

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AUC, area under the curve; DZ, dizygotic; HOMA, homeostasis model assessment; IGT, impaired glucose tolerance; MZ, monozygotic; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PPAR, peroxisome proliferator–activated receptor.

The association between birth anthropometry and glucose intolerance and/or type 2 diabetes has consistently been demonstrated in several epidemiological studies among singletons (1). However, it has been proposed that the association may be due to a genotype leading to both low birth weight and subsequent development of diabetes in adult life (2). Among a homogenous population-based twin sample, we have recently reported elevated levels of plasma glucose and insulin after oral glucose ingestion among monozygotic (MZ) compared with dizygotic (DZ) twins (3), supporting a role of the intrauterine environment on glucose homeostasis given the more adverse intrauterine conditions characterizing MZ pregnancies (4). Although other studies have not replicated these findings in younger twin populations (5,6), we have recently demonstrated a lower in vivo insulin action, measured by the euglycemic hyperinsulinemic clamp technique, among elderly MZ compared with DZ twins (7).

A recent study reported a higher frequency of the silent C→T substitution in exon 6 of *PPARγ* among DZ relative to MZ twins of different ethnic origins (8). The authors concluded that this silent mutation may be linked to DZ twinning and involved in intrauterine survival of DZ twins. Although the frequency of the Pro12Ala polymorphism was similar among MZ and DZ twins, some evidence of transmission disequilibrium was reported for the combination of the two different polymorphisms from the parents of the DZ twins. Another recent study found no linkage between *PPARγ* and dizygotic twinning. The study, however, did not report allele or genotype frequencies among MZ and DZ twins (9).

Peroxisome proliferator–activated receptors (PPARs) are members of the nuclear hormone receptor family of transcription factors and are involved in adipocyte differentiation and gene expression. The common Pro12Ala polymorphism of *PPARγ2*, initially identified by Yen et al. (10), has inconsistently been associated with protection against type 2 diabetes and associated diabetic traits, including insulin resistance (11–18). One study has reported an association between the C→T substitution in exon 6 and a reduced risk of cardiovascular disease (19). No studies have directly addressed the phenotypic appear-

ance according to plasma glucose and insulin profiles of the silent C→T substitution in exon 6.

Given the important role of PPARγ in glucose homeostasis, it is tempting to speculate that differences in *PPARγ* polymorphisms, and not the intrauterine environment, may explain our previous findings of increased plasma glucose and insulin profiles after oral glucose ingestion in MZ compared with DZ twins. Thus, in the present study we report frequencies of the Pro12Ala and C→T polymorphism among a homogeneous population-based sample of Danish Caucasian MZ and DZ twins. Furthermore, we report the phenotypic appearance according to plasma glucose and insulin profiles of the polymorphisms, and in particular, we have determined whether any differences in genotype frequencies can explain our findings of different metabolic profiles among MZ and DZ twins (3).

**Subjects.** The twin sample was population-based and ascertained through the Danish twin register as described previously (20). A total of 303 (125 MZ and 178 DZ) twin pairs participated in the clinical examination, which involved a standard oral glucose tolerance test (OGTT) and anthropometric measurements. Zygosity classification was established using the similarity method (1). Genotyping of the twins revealed a misclassification of three pairs (genotypic discordant MZ pairs), who were excluded from further analysis because of uncertain zygosity classification. DNA was not available from 46 twins, leaving a total of 554 twins in the present study; 210 MZ (90 pairs and 30 single twins) and 344 DZ (168 pairs; 8 single twins).

The study was approved by the regional ethical committees and was conducted according to the principles of the Helsinki Declaration.

## RESEARCH DESIGN AND METHODS

The clinical examination, including an OGTT and anthropometric measures, was performed as previously described (1,20). Plasma glucose and insulin concentrations were measured and analyzed as described (1,20). Incremental glucose and insulin areas under the curves (AUCs) were calculated using the trapezoidal method. Measures of insulin resistance were calculated from fasting plasma glucose and insulin concentrations using homestasis model assessment (HOMA) (21). Type 2 diabetes and impaired glucose tolerance were defined according to the 1998 World Health Organization criteria.

The genetic analyses were performed on genomic DNA isolated from human leukocytes. The Pro12Ala variant was genotyped as previously described (22). The exon 6 C→T silent histidine variant was genotyped with primers 5′-TGT GAA GCC CAT TGA AGA CA-3′ and 5′-GAG CGG GTG AAG ACT CAT GT-3′ (derived from EMBL no. AB005526) and carried out in a volume of 25 μl containing 100 ng genomic DNA, 1× PCR buffer, 0.2 μmol/l of each primer, 0.2 mmol/l dNTP, 1.4 units AmpliTaq Gold polymerase (Applied Biosystems), and 3 mmol/l $MgCl_2$. The cycling program was a denaturation step at 95°C for 15 min followed by 40 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 60 s, with a final elongation step at 72°C for 9 min using a GeneAmp 9600 thermal cycler (Perkin Elmer). The PCR product was digested overnight at 37°C with two units of *Nla*III (New England Biolabs), and fragments were separated on a 4% agarose gel. The concordance rates between the PCR–restriction fragment–length polymorphism assay used for genotyping and sequencing or repeat genotyping was 100%.

**Statistical analyses.** Fischer's exact test was applied to compare allele frequencies and genotype distributions in subgroups of twins. Only one random twin from each twin pair was included in the analysis. The test for Hardy-Weinberg equilibrium was performed as described previously (23).

Since MZ twins share their entire genome and DZ twins share half of their genome, one may question the supposition that the twins are independent observations. Accordingly, we have performed an ANOVA for each phenotypic parameter in which we adjusted for the inter–twin-pair relationship. The full ANOVA model includes a random effect term for twin-pair membership and fixed effect terms for PPARγ and zygosity and their interaction, i.e., different effects of PPARγ in MZ and DZ twins. The interaction effect was not significant, and the tests of zygosity and genotype were made in the model without interaction terms. In addition, it was investigated by paired comparisons whether the Pro12Ala and the Ala12Ala genotypes could be pooled. The comparisons showed no significant differences, and therefore these two genotypes were combined. The statistical analyses were performed with PROC MIXED of the SAS/STAT system (Version 8.2, SAS Institute ). Initial descriptive data analysis showed that all variables except waist-to-hip ratio exhibited markedly right-skewed distribution. Therefore, a transformation was considered necessary for application of the mixed ANOVA, and a logarithmic transformation was found to give a much better approximation to a symmetric Gaussian distribution. The means are reported as the antilog of the means of the log-transformed values, and SE was calculated using the standard error of the log-transformed mean. Comparisons of metabolic and anthropometric phenotype data within the genotypically discordant DZ twin pairs were performed using the parametric paired *t* test for normally distributed data and the Wilcoxon's signed-rank test for skewed data. A *P* value ≤0.05 was considered significant. Due to the fact that a meta-analysis of all published papers strongly supports that the Pro12Ala polymorphism (22) represents a significant type 2 diabetes and insulin resistance gene, we have found it reasonable not to correct for multiple comparisons when analyzing the effect of this polymorphism on glucose homeostasis.

TABLE 1
Genotype distribution and allele frequencies of the Pro12Ala and exon 6 C→T polymorphisms of PPARγ according to zygosity

| | MZ | DZ | *P* |
|---|---|---|---|
| Pro12Ala | | | |
| Pro/Pro | 90 (76) | 132 (77) | |
| Pro/Ala | 25 (21) | 36 (21) | 0.7 |
| Ala/Ala | 4 (3) | 3 (2) | |
| Allele frequency | 13.9 (9.5–18.3) | 12.3 (8.8–15.8) | 0.6 |
| Exon 6 | | | |
| C/C | 89 (75) | 142 (83) | |
| C/T | 30 (25) | 27 (16) | 0.06 |
| T/T | 0 (0) | 2 (1) | |
| Allele frequency | 12.6 (8.4–16.8) | 9.1 (6.0–12.1) | 0.2 |

The MZ ($n$ = 119) and DZ ($n$ = 171) twin groups include only one (random) twin from each pair and single twins. Genotype distribution is presented as $n$ (%); allele frequency is presented as frequency (95% CI).

## RESULTS

**Genotype and allele frequencies according to zygosity.** The distribution of both polymorphisms was in Hardy-Weinberg equilibrium among both MZ and DZ twins. The allele and genotype frequencies were similar in both MZ and DZ twins for each polymorphism (Table 1). However, the genotype distribution of the exon 6 polymorphism was nearly significantly different between MZ and DZ twins ($P$ = 0.06) (Table 1). The degree of allele sharing among the DZ twins (phenotype, DZ twin) was 0.91 (SD 0.20) compared with an expected value of 0.90 ($P$ = 0.94).

**Genotype and allele frequencies according to glucose tolerance.** There was a near significant difference in the genotype distribution according to glucose tolerance status, with a higher proportion of the Pro/Pro genotype among twins with type 2 diabetes and impaired glucose tolerance (IGT) compared with the group of normal glucose tolerant twins ($P$ = 0.07). Twins with type 2 diabetes and the combined group of twins with type 2 diabetes and IGT had a significantly lower frequency of the minor Ala allele compared with twins with normal glucose tolerance (NGT). No differences in allele frequency of the minor T-allele of the exon 6 polymorphism according to glucose tolerance were observed (Table 2).

**Phenotypic appearances of the two variants in the *PPARγ* gene among MZ and DZ twins.** Both MZ and DZ

TABLE 2
Genotype distribution and allele frequencies of the Pro12Ala and exon 6 C→T polymorphisms of *PPARγ* according to glucose tolerance status

| | Type 2 diabetes | IGT | NGT | *P* |
|---|---|---|---|---|
| *n* | 32 | 54 | 188 | |
| Pro12Ala | | | | |
| Pro/Pro | 30 (94) | 45 (83) | 141 (75) | |
| Pro/Ala | 2 (6) | 8 (15) | 41 (22) | 0.07 (0.07) |
| Ala/Ala | 0 (0) | 1 (2) | 6 (3) | |
| Allele frequency | 3.1 (−1.1 to 7.4) | 9.3 (3.8–14.7) | 15.2 (11.8–18.6) | 0.01 (0.01) |
| Exon 6 | | | | |
| C/C | 26 (81) | 45 (83) | 148 (79) | |
| C/T | 6 (19) | 8 (15) | 38 (20) | 1.0 (0.5) |
| T/T | 0 (0) | 1 (2) | 1 (1) | |
| Allele frequency | 9.4 (2.2–16.5) | 9.3 (3.8–14.7) | 10.7 (7.6–13.8) | 1.0 (0.7) |

Only one (random) twin from each pair and single twins are included in the analysis. *P* indicates the *P* values in the comparison of type 2 diabetes versus NGT and abnormal glucose tolerance (type 2 diabetes plus IGT) versus NGT, respectively. Genotype distribution is presented as *n* (%); allele frequency is presented as frequency (95% CI).

carriers of the Pro and Ala allele had similar anthropometry. The Ala allele was associated with a significantly lower glucose concentration after 120 min and AUC for glucose compared with the Pro allele. Furthermore, the fasting and 120-min plasma insulin concentrations together with the AUC for insulin were significantly lower among carriers of the Ala allele compared with the Pro allele carriers. Finally, the carriers of the Ala allele were significantly less insulin resistent (HOMA index) compared with the carriers of the Pro allele (Table 3). The exon 6 polymorphism was not associated with measures of anthropometry, plasma glucose, or insulin concentrations (*P* values between 0.2 and 0.9) among MZ and DZ, respectively (data not shown).

**Metabolic profiles according to zygosity.** Independent of genotype, the MZ twins had significantly higher 30-min plasma insulin concentrations and AUC for insulin compared with DZ twins (Table 3). Furthermore, there was a tendency toward higher 30-min plasma glucose concentrations ($P = 0.09$) and AUC for glucose ($P = 0.10$) among MZ twins compared with DZ twins.

**Twins discordant for the variant genotypes.** We identified 27 dizygotic twin pairs discordant for the Pro12Ala polymorphism (i.e., Pro/Pro versus Pro/Ala). Twins homozygous for the Pro allele had significantly higher 120-min plasma insulin concentrations ($P = 0.007$) and near significantly higher 120-min plasma glucose concentrations ($P = 0.06$) and AUC for plasma insulin ($P = 0.08$) (Table 4). Among the 27 dizygotic twin pairs discordant for the exon 6 C→T polymorphism (i.e., C/C versus C/T), no differences in phenotypes were observed (data not shown).

## DISCUSSION

Several recent studies reported an association between the Ala allele of the codon 12 polymorphism and reduced risk of diabetes (12–15,22), decreased insulin resistance (13,16–18,22), and low (16) or high BMI (24). However, not all studies have been able to replicate these associations (25–31). Our finding of a significantly higher frequency of the Ala allele among twins with NGT provides further evidence in favor of an association between the Ala allele and a reduced risk of type 2 diabetes and/or IGT. This is

TABLE 3
Phenotypic appearance of the Pro12Ala polymorphism of *PPARγ* among MZ and DZ twins

| | Pro/Pro | | Pro/Ala + Ala/Ala | | *P* (ANOVA) | |
|---|---|---|---|---|---|---|
| | MZ | DZ | MZ | DZ | Zygosity | Genotype |
| Subjects (families) | 161 ± 90 | 268 ± 135 | 47 ± 29 | 77 ± 41 | | |
| Age (years) | 67.2 ± 0.4 | 66.1 ± 0.4 | 66.9 ± 1.0 | 66.8 ± 0.8 | | |
| BMI (kg/m$^2$) | 25.9 ± 0.4 | 25.7 ± 0.3 | 25.0 ± 0.6 | 25.1 ± 0.4 | 0.70 | 0.09 |
| Waist-to-hip ratio | 0.877 ± 0.008 | 0.868 ± 0.007 | 0.871 ± 0.015 | 0.862 ± 0.010 | 0.37 | 0.50 |
| Glucose (mmol/l) | | | | | | |
| 0 min | 5.97 ± 0.11 | 5.99 ± 0.08 | 6.04 ± 0.20 | 5.72 ± 0.13 | 0.62 | 0.22 |
| 30 min | 9.62 ± 0.20 | 9.38 ± 0.15 | 9.68 ± 0.36 | 8.94 ± 0.25 | 0.09 | 0.26 |
| 120 min | 7.36 ± 0.28 | 7.26 ± 0.19 | 6.52 ± 0.46 | 6.30 ± 0.30 | 0.65 | 0.0034 |
| AUC | 272 ± 14 | 245 ± 10 | 219 ± 26 | 204 ± 19 | 0.096 | 0.016 |
| Insulin (pmol/l) | | | | | | |
| 0 min | 42.9 ± 2.2 | 39.3 ± 1.5 | 32.8 ± 2.9 | 35.6 ± 2.2 | 0.42 | 0.0078 |
| 30 min | 274 ± 18 | 230 ± 10 | 241 ± 27 | 221 ± 17 | 0.029 | 0.35 |
| 120 min | 249 ± 19 | 223 ± 12 | 169 ± 20 | 164 ± 14 | 0.25 | <0.0001 |
| AUC | 24,302 ± 1,548 | 20,490 ± 1,022 | 20,320 ± 1,926 | 17,546 ± 1,313 | 0.016 | 0.021 |
| HOMA | 1.90 ± 0.11 | 1.75 ± 0.07 | 1.47 ± 0.15 | 1.51 ± 0.11 | 0.38 | 0.0075 |

Data are means ± SE.

TABLE 4
Dizygotic twinpairs discordant for the Pro/Ala polymorphism of *PPARγ*

| | Pro/Pro | Pro/Ala | *P* |
|---|---|---|---|
| *n* | 27 | 27 | |
| BMI | 24.9 ± 0.8 | 25.6 ± 0.9 | 0.43 |
| Waist-to-hip ratio | 0.88 ± 0.02 | 0.87 ± 0.02 | 0.79 |
| Glucose | | | |
| 0 min | 5.8 ± 0.1 | 5.6 ± 0.1 | 0.12 |
| 30 min | 9.5 ± 0.3 | 9.2 ± 0.3 | 0.40 |
| 120 min | 7.2 ± 0.4 | 6.2 ± 0.3 | 0.06 |
| AUC | 282.9 ± 23.1 | 243.1 ± 22.8 | 0.19 |
| Insulin | | | |
| 0 min | 41.7 ± 3.6 | 36.7 ± 3.9 | 0.19 |
| 30 min | 288.3 ± 36.8 | 291.5 ± 45.6 | 0.72 |
| 120 min | 247.4 ± 29.7 | 161.6 ± 19.9 | 0.007 |
| AUC | 24,053 ± 2,761 | 20,910 ± 2,925 | 0.08 |
| HOMA | 1.8 ± 0.2 | 1.6 ± 0.2 | 0.13 |

Data are means ± SE.

illustrated by the lower plasma glucose and insulin concentrations during OGTT together with lower HOMA insulin resistance among carriers of the Ala allele compared with the noncarriers. Since measures of obesity were similar in carriers of the different genotypes, obesity does not appear to be a confounding factor in the observed metabolic differences between the different genotypes. Finally, the lower 120-min plasma glucose and insulin concentrations and AUC for insulin in DZ carriers of the Ala allele, compared with their discordant cotwin carrying the Pro allele, further supports this finding.

In the present study, we found no association between the exon 6 C→T polymorphism and variables related to type 2 diabetes or insulin resistance.

The allele frequencies of the exon 6 T-allele (MZ 12.6% and DZ 9.1%) among the present twin population are not in line with the findings of Busjahn et al. (8) (MZ 9% and DZ 19%). Moreover, the degree of allele sharing among the DZ twins did not differ from the expected value for allele sharing. Therefore, our data does not provide any major support for the idea that the silent C→T substitution in exon 6 of the *PPARγ* gene may be linked to DZ twinning. The present study population, including 550 subjects, is a homogenous population-based sample of Danish Caucasian twins, while the population in the study by Busjahn et al. (8) included 606 subjects from three different European countries. Among these subjects 100 DZ twins were from Finland, a population that exhibits a higher allele frequency of the Pro12Ala polymorphism than other European populations (15). If the exon 6 variant cosegregates with the Pro12Ala variant, the discrepancies in these results could be explained by differences in ethnicity. However, the associations in the study by Busjahn et al. (8) were in fact even more pronounced in the non-Finish populations, which indicates that other ethnic differences or unknown factors may explain the different results.

We observed similar allele frequencies of both *PPARγ* polymorphisms among MZ and DZ twins. These findings alone indicate that the metabolic differences among MZ and DZ twins cannot be explained by differences in the *PPARγ* genotypes. When comparing anthropometry and measures of OGTT plasma glucose and insulin concentrations between MZ and DZ twins, MZ twins had significantly higher 30-min and AUCs for plasma insulin compared with DZ twins, independent of genotype. The higher concentrations of 30-min and AUCs for plasma glucose during the OGTT among MZ compared with DZ twins did not reach the level of statistical significance. This can be explained by the smaller number of twins included in the present study and/or the fact that we performed corrections for intrapair relationships. Although the possibility remains that other unknown genotypes may play a role, we still believe that the differences in metabolic profiles between MZ and DZ twins might be explained by differences in their respective intrauterine environment, in accordance with the thrifty phenotype hypothesis (1).

In conclusion, the differences in plasma insulin profiles during OGTT among MZ and DZ twins, the latter being less insulin resistant, cannot be explained by differences in frequencies of the *PPARγ* variants. The differences may therefore, as previously reported, be due to intrauterine environmental factors independent of genotype. However, with the present study we provide additional evidence in favor of an association between the Pro12Ala variant and reduced risk of diabetes and reduced insulin resistance. Therefore, our study simultaneously provides evidence for a role of both the intrauterine environment and the thrifty phenotype hypothesis and for genetics and the thrifty genotype hypothesis in the etiology of insulin resistance and perhaps glucose intolerance in twins.

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