Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS   Check for updates

Role of peroxisome proliferator-activated receptor gamma Pro12Ala polymorphism in human adipose tissue: assessment of adipogenesis and adipocyte glucose and lipid turnover

Prasad G. Kamble[a], Maria J. Pereira[a], Stefan Gustafsson[b], Per Lundkvist[a], Casimiro Castillejo-López[b], Tove Fall[b], Erik Ingelsson[b,c]*, and Jan W. Eriksson[a]*

[a]Department of Medical Sciences, Clinical Diabetes and Metabolism, Uppsala University, Uppsala, Sweden; [b]Department of Medical Sciences, Molecular Epidemiology and Science for Life Laboratory, Uppsala University, Uppsala, Sweden; [c]Department of Medicine, Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA, USA

ABSTRACT

The protective mechanisms of peroxisome proliferator-activated receptor gamma (PPARγ) Pro12Ala polymorphism in type 2 diabetes (T2D) are unclear. We obtained subcutaneous adipose tissue (AT) before and 3 h after oral glucose (OGTT) in carriers and non-carriers of the Ala allele (12 Pro/Pro, 15 Pro/Ala, and 13 Ala/Ala). Adipogenesis, adipocyte glucose uptake and lipolysis as well as PPARγ target gene expression were investigated and compared between the genotype groups. During fasting and post-OGTT, neither basal nor insulin-stimulated adipocyte glucose uptake differed between genotypes. Compared to fasting, a decreased hormone-sensitive lipase gene expression in Pro/Pro ($p < 0.05$) was accompanied with a higher antilipolytic effect of insulin post-OGTT ($p < 0.01$). The adipocyte size was similar across groups. Preadipocyte differentiation rates between Pro/Pro and Ala/Ala were unchanged. In conclusion, no major differences in AT differentiation, glucose uptake, lipolysis or expression of PPARγ target genes were observed between different PPARγ Pro12Ala genotypes. Albeit small, our study may suggest that other pathways in AT or effects exerted in other tissues might contribute to the Pro12Ala-mediated protection against T2D.

Introduction

The peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-induced nuclear transcription factor that regulates adipogenesis and coordinates metabolic functions to maintain glucose and lipid homeostasis and improves insulin sensitivity.[1] Consequently, synthetic PPARγ ligands have been used in the management of type 2 diabetes (T2D).[2] Two *PPARγ* isoforms, *PPARγ1*, and *PPARγ2* exist due to mRNA splicing.[3] The difference between these two isoforms is the presence of an additional 28 amino acids towards the N-terminal of *PPARγ2*, conferring PPARγ2 protein a higher ligand-independent transactivation ability than PPARγ1.[4]

The common Pro12Ala polymorphism affecting PPARγ2 was identified through screening of the coding region in diabetes patients and causes substitution of the amino acid proline to alanine at the 12th codon in exon B of PPARγ2.[5] The frequency of Ala 12 variant varies from 4% to 28% with higher prevalence among people of northern European ancestry.[6] Functionally, the product of this gene variant has reduced affinity and transcriptional activity, but is associated with improved insulin sensitivity and reduced risk of T2D through an undefined mechanism.[6–8] This high frequency of Ala allele contributes largely to its protective effect at the population level despite its modest effect on diabetes prevention.[9] Several human studies have indicated an association of Pro12Ala polymorphism with glucose and lipid metabolism,[10–14] although the associations are discrepant between different ethnic subgroups.[15]

Given the exclusive expression of PPARγ2 in adipose tissue (AT),[16] it is plausible to speculate that any protective effect mediated by the Ala variant would be secondary to shift in AT metabolism. However, limited studies have been performed to address mechanisms underlying protective effects of the Ala variant. The present study employed a genotype-based recall (GBR) approach to recruit subjects based on their genotype status for the minor (Ala) allele and studied its effect on the expression of PPARγ target genes in adipose tissue, adipocyte glucose uptake and lipolysis regulation and adipogenesis.

Results

Effect of Pro12Ala on target gene expression

To assess whether reduced ligand binding due to Ala12 substitution affects the *in vitro* transcriptional activity of PPARγ2, we measured expression levels of PPARγ target genes in freshly snap frozen subcutaneous AT obtained upon fasting and after 3 h OGTT (Figure 1A and B). The genes that are known to be regulated by PPARγ and are involved in various aspects of AT biology were selected. The genes we selected are involved in adipogenesis (cluster of differentiation 36 (*CD36*), CAAT enhancer binding protein alpha (*CEBP alpha*), fatty acid binding protein 4 (*FABP4*), adiponectin (*ADIPOQ*) and *PPARγ*), glucose uptake (insulin receptor substrate 1 and 2 (*IRS1, IRS2*) and glucose transporter 4 (*GLUT4*)), lipid metabolism (hormone sensitive lipase (*HSL*), fatty acid synthase (*FAS*) and lipoprotein lipase (*LPL*)), proinflammatory cytokine (tumor necrosis factor alpha (*TNF-alpha*)), and also in thermogenesis (uncoupling protein 1((*UCP1*)). On fasting, only *HSL* expression was higher by about 50% in Pro/Pro and Pro/Ala, compared to Ala/Ala ($p < 0.01$, Figure 1B)

Given previously shown regulation of PPARγ2 by nutrients,[17] we also evaluated whether presence of Pro12Ala differentially modulates expression of PPARγ target genes in response to OGTT. However, we did not observe any significant difference in the expression of genes tested (*PPARγ, GLUT4, FAS*, and *LPL*) between three genotype groups (Figure 1B). When compared to fasting, only *HSL* gene expression was significantly reduced post-OGTT by about 35% in Pro/Pro and Pro/Ala group ($p < 0.05$, Figure 1B).

Effect of Pro12Ala on adipocyte glucose uptake

AT obtained upon fasting and post-OGTT from different groups of carrier status of the Pro12Ala gene variant was digested using collagenase and isolated adipocytes were used to study the basal and insulin-stimulated glucose uptake capacity. On fasting and post-OGTT, the basal glucose uptake did not change between genotypes (Figure 2A). No significant difference was observed in the sub-maximal (25 μU/ml) and maximal (1000 μU/ml) insulin-stimulated glucose uptake both on fasting and post-OGTT between the three genotype groups (Figure 2B).

Effect of Pro12Ala on adipocyte lipolysis

Next, we studied basal, isoproterenol-stimulated (0.5 μM) and antilipolytic effect of insulin (0.1 to 100 μU/ml) on isolated adipocytes obtained on fasting and post-OGTT
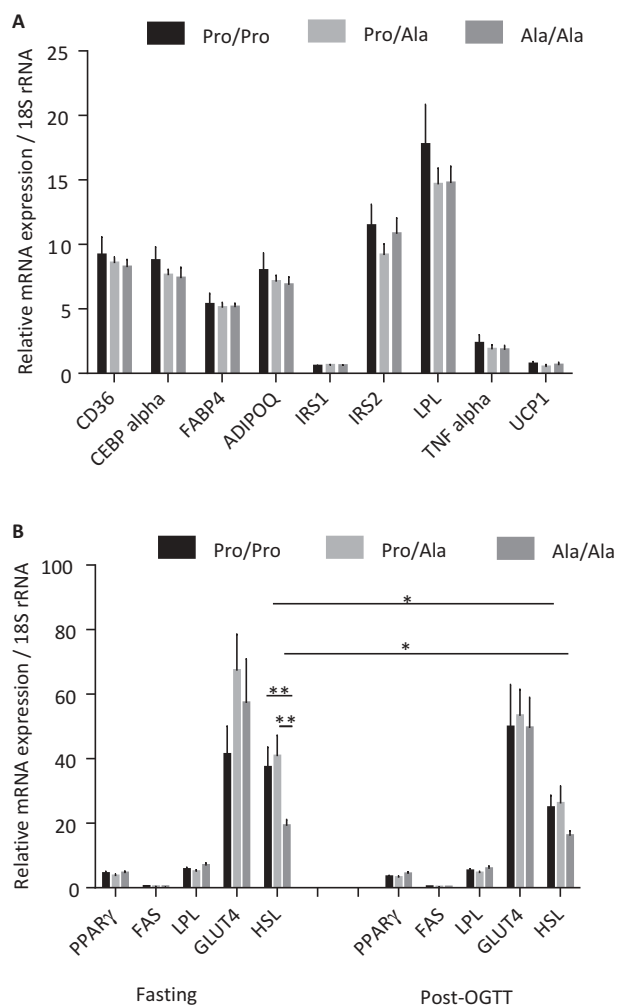
Figure 1. Effect of Pro12Ala on target gene expression. The subcutaneous adipose tissue obtained upon fasting and post-OGTT from different genotype groups was snap frozen, RNA was isolated and reverse-transcribed and gene expression was analyzed using the real-time quantitative PCR (A) Expression of genes on fasting. (B) Expression of genes on fasting and post-OGTT. *18S rRNA* was used as an endogenous control. All data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

ADIPOQ: adiponectin, *CD36*: cluster of differentiation 36, *CEBP alpha*: CAAT enhancer binding protein alpha, *FABP4*: fatty acid binding protein 4, *FAS*: fatty acid synthase, *GLUT4*: glucose transporter 4, *HSL*: hormone-sensitive lipase, *IRS1 and 2*: insulin receptor substrate 1 and 2, *LPL*: lipoprotein lipase, OGTT: oral glucose tolerance test, *PPARγ*: peroxisome proliferator-activated receptor gamma, *TNF alpha*: tumor necrosis factor alpha, *UCP1*: uncoupling protein 1.

from carriers with different Pro12Ala gene variants. Amount of glycerol released into the medium at the end of the reaction was taken as an index of lipolysis. On fasting, the antilipolytic effect of insulin did not differ between the three genotype groups (Figure 3A). Post-OGTT, the antilipolytic effect of insulin was significantly higher in Pro/Pro group than Pro/Ala (insulin 10 μU/ml and 100 μU/ml, $p < 0.01$ and $p < 0.05$, respectively), but
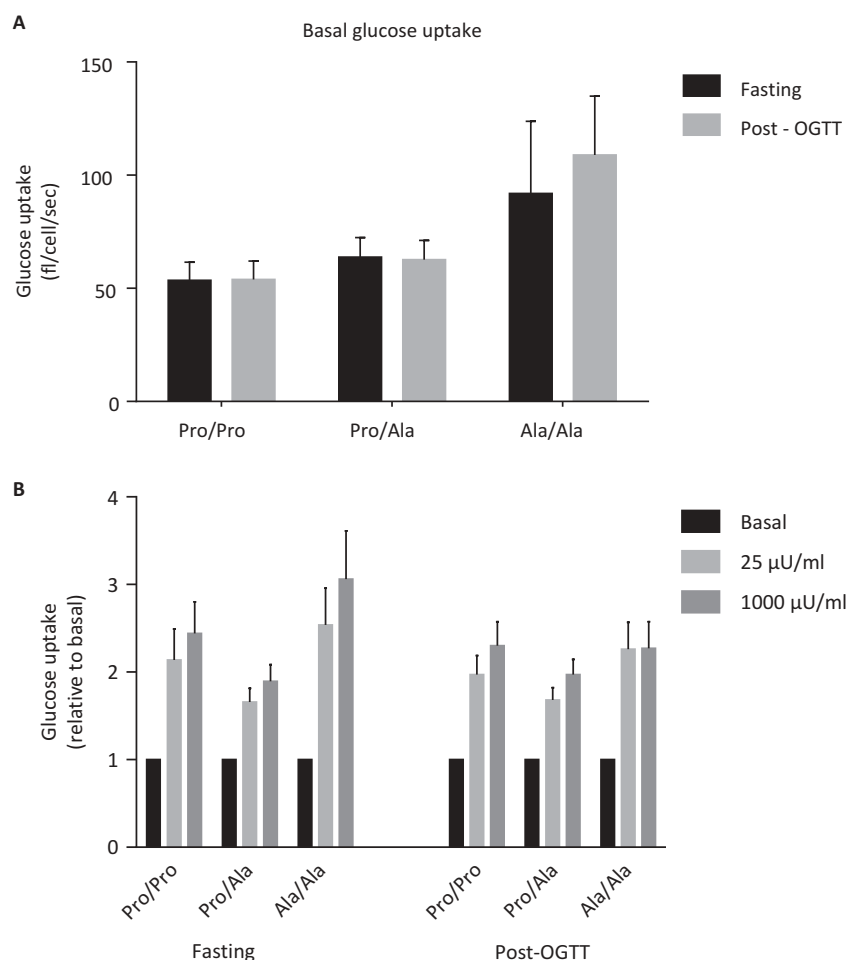
A

Basal glucose uptake

B

Figure 2. Glucose uptake in adipocytes obtained from Pro/Pro (n = 12), Pro/Ala (n = 15) and Ala/Ala (n = 13) on fasting and after 3 h OGTT. Adipose tissue was digested with collagenase and isolated subcutaneous adipocytes were incubated with or without insulin for 15 minutes followed by incubation with D-[U-^{14}C] glucose for next 45 minutes to measure (A) basal, (B) submaximal (25 µU/ml) and maximal (1000 µU/ml) insulin-stimulated glucose uptake, respectively. (n = 12 Pro/Pro, 15 Pro/Ala and 13 Ala/Ala). All data are presented as mean ± SEM.

not when compared to Ala/Ala (Figure 3B). The basal and isoproterenol-stimulated lipolysis (relative to basal which was set to one) did not differ significantly between different genotype groups, both on fasting and after an OGTT (Figure 3C and D). In addition, when compared to fasting, isoproterenol-stimulated lipolysis after OGTT was significantly reduced in Ala carriers (p < 0.05) (Figure 3D).

Adipocyte size

The diameter of 100 adipocytes was measured from random areas chosen under the microscope. There was no significant difference in the mean cell diameter of isolated subcutaneous adipocytes obtained from all three different genotype groups (Figure 4).

Effect of Pro12Ala on preadipocytes differentiation capacity

After AT digested with collagenase, isolated preadipocytes from Pro/Pro (n = 6) and Ala/Ala (n = 7) were used to study their adipogenic potential. The degree and rate of adipogenesis were assessed by quantifying the amount of lipid accumulation at the end, and measuring the expression of adipogenic markers at different time points during differentiation, respectively. The percent differentiation measured by lipid accumulation on the 14th day of differentiation did not differ between the two groups (Figure 5A). Furthermore, differentiation rate assessed by measuring the expression levels of adipogenic markers (*CEBP alpha, PPARγ,* and *ADIPOQ*) at different times during differentiation also did not change between the genotypes (Figure 5B). In addition, we also analyzed the expression levels of
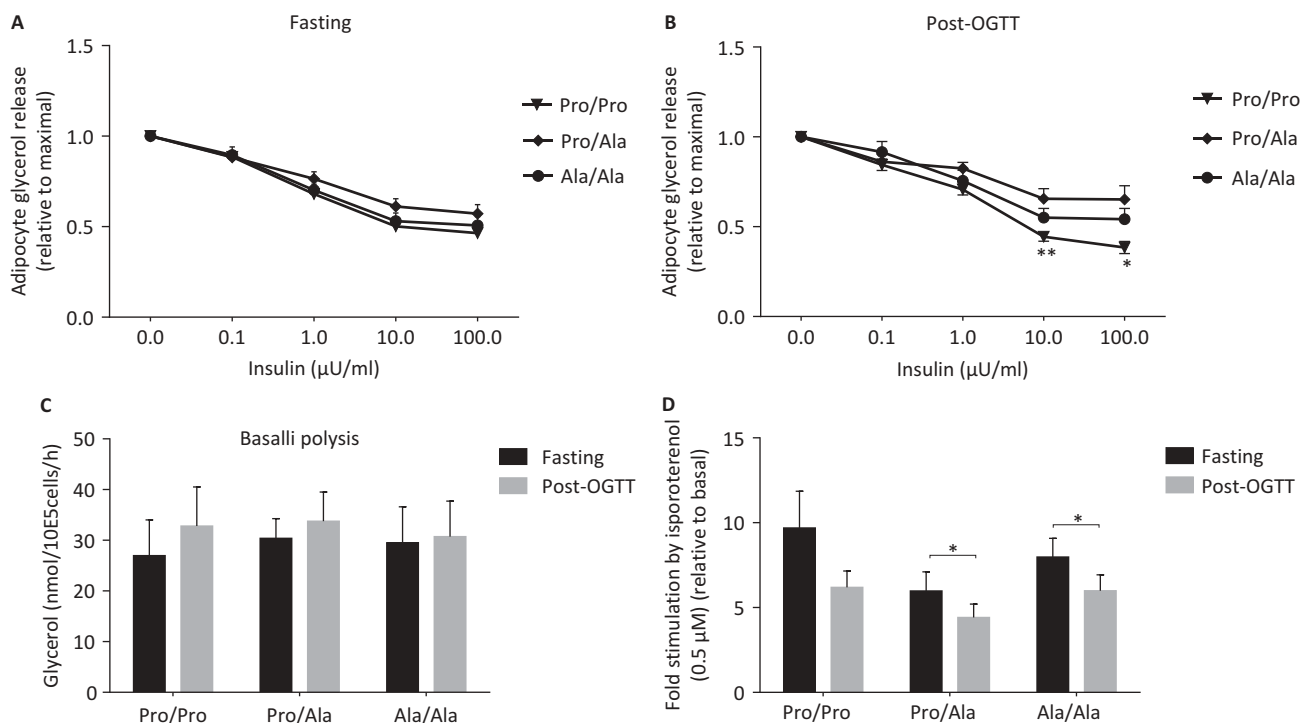
Figure 3. Lipolysis in adipocytes from subjects with Pro/Pro (n = 12), Pro/Ala (n = 15) and Ala/Ala (n = 13) on fasting and after 3 h OGTT. Subcutaneous adipocytes were isolated as described in "Methods". The cells suspension was supplemented without (basal) or with (isoproterenol (0.5 μM), and insulin (0.1 to 100 μU/ml) in the presence of isoproterenol (0.5 μM) for 2 hours. The amount of glycerol released into the medium was measured and taken as an index of lipolysis (A) On fasting, insulin-mediated inhibition of isoproterenol-stimulated lipolysis did not differ between the three genotype groups. (B) Post-OGTT, the antilipolytic effect of insulin was significantly higher in a Pro/Pro group than Pro/Ala but not when compared to Ala/Ala group. (C) Basal lipolysis shown as the amount of glycerol released under a non-stimulated condition on fasting and post-OGTT. (D) Fold stimulation by isoproterenol (0.5 μM) relative to basal (set to 1) on fasting and post-OGTT. The results in (A) and (B) are shown as glycerol release relative to maximal (isoproterenol-stimulated lipolysis) set to 1 (n = 12 Pro/Pro, 15 Pro/Ala, and 13 Ala/Ala). All data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. OGTT: oral glucose tolerance test.

markers of mitochondrial biogenesis during adipogenesis (*CPT1 alpha and beta and PGC1 alpha*); however, no difference was observed between groups (Figure 5B).

Discussion

The minor Ala allele at the 12th position of PPARγ2 gene has been shown to reduce transcriptional activity of its protein and offers protection against the development of T2D. However, underlying cellular mechanisms remained elusive. Under normal physiological conditions, the expression of PPARγ2 isoform is restricted to AT.[16,18] Therefore effects mediated by the Ala variant are expected to primarily occur in AT, and it would be suspected that this involves changes in AT metabolism. Thus, we obtained subcutaneous AT from carriers with 0, 1 or 2 copies of the Ala variant on fasting and post-OGTT, and studied its effects on the expression of PPARγ target genes in AT, *ex vivo* adipocyte glucose uptake and lipolysis assays and cell size.

Lastly, for the first time, we studied the effect of Pro12Ala on human preadipocytes differentiation.

On fasting, *HSL* gene expression was lower in Ala/Ala; a difference that was attenuated post-OGTT. Next, no difference was seen between the three genotype groups on adipocyte glucose uptake capacity tested before or post-OGTT. On fasting, the adipocyte lipolysis did not change between study groups. However, post-OGTT, lipolysis inhibition by insulin was higher in the Pro/Pro group. The adipocyte size and preadipocyte differentiation also showed no major change between the genotype groups.

PPARγ2 controls AT metabolism by regulating the expression of its target genes, hence a decrease in its ligand binding and transactivation capacity due to Pro12Ala could alter metabolic activities of AT. We studied the expression of genes that have a role in adipogenesis, glucose and lipid metabolism and are regulated by PPARγ2. However, we did not find any difference in expression of genes between the three
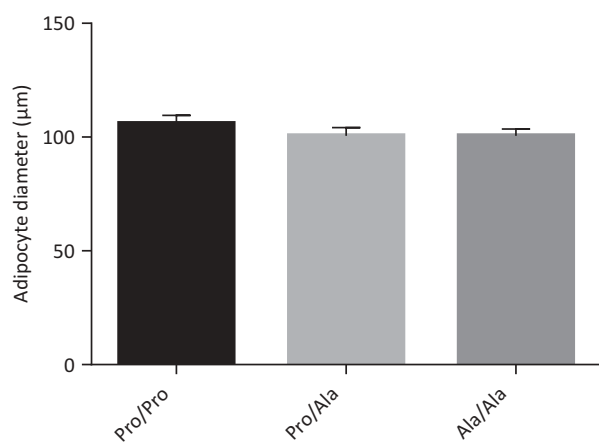
Figure 4. Adipocyte diameter between the three genotype groups. Adipose tissue was digested with collagenase and diameter of 100 isolated adipocytes was measured from randomly selected areas on a siliconized glass slide and showed as the mean adipocyte diameter. No difference was observed in the mean cell diameter of subcutaneous adipocytes obtained from the three different genotype groups. All data are presented as mean ± SEM.

genotype groups, suggesting that Ala variant has no major effect on PPARγ2 transcriptional activity. Our findings are in agreement with a previous report.[19] In contrast, Pihlajamaki et al.[20] found a significantly increased expression of *PPARγ* in Ala/Ala group compared to Pro/Pro. Of note, a study by[20] recruited only males. Nevertheless, in our study population, we did not see any sex-related difference in the expression of *PPARγ* (data not shown). PPARγ activity has been shown to be regulated by nutritional factors.[17] Thus, we analyzed the expression of *PPARγ* and its target genes in AT obtained after OGTT, but similar to fasting, no change was observed in the mRNA levels of genes assessed, except for *HSL* as described below.

Thiazolidinediones have been shown to increase adipocyte glucose uptake.[21,22] We hypothesized that an increase in adipocyte glucose uptake could be a factor causing an insulin sensitization in Ala 12 carriers. However, the adipocyte glucose uptake capacity did not change between the three genotype groups. In contrast, a previous study in PPARγ2 Pro12Ala knock-in mice showed an increase in insulin-stimulated glucose uptake in white AT on chow diet.[23] The discrepancy between experimental models could explain the different results observed. In line with our findings, a study by Vänttinen et al.[24] showed no difference in glucose uptake in human subcutaneous AT measured *in vivo* under the hyperinsulinemic-euglycemic clamp, suggesting that Pro12Ala might not influence glucose utilization by AT in humans. Another study in T2D patients reported similar results.[25] Vänttinen et al.[24] found a higher glucose uptake in skeletal muscle in non-obese

Pro12Ala subjects than wild-type carriers. As shown previously, an ectopic expression of PPARγ2 in muscle and liver under over nutrition and genetic obesity,[17,26] may have contributed to an observed glucose uptake in skeletal muscle under clamp condition.[24] This further implies that tissues other than adipose might be important in mediating insulin sensitivity in Pro12Ala subjects, whereas AT, on the other hand, regulates the release of free fatty acids (FFA) and adipokines to facilitate improved insulin sensitivity. Indeed, a previous study has shown a higher antilipolytic activity during the hyperinsulinemic-euglycemic clamp in Ala12 carriers.[6] However, the cellular mechanisms are unknown. This prompted us to study whether adipocyte lipolysis regulation is altered between different carriers of Pro12Ala variant.

On fasting as well as after OGTT, neither basal nor isoproterenol-stimulated lipolysis changed in adipocytes from the three different groups. In addition, upon fasting, antilipolytic effect of insulin also did not differ between genotypes. However, a higher antilipolytic effect of insulin was seen post-OGTT in the Pro/Pro. We have no clear explanation for the observed unexpected effect of OGTT on lipolysis. Neither did we find any studies assessing *ex vivo* adipocyte lipolysis post-OGTT. Nevertheless, a significantly low stimulation of lipolysis post-OGTT in Pro/Ala and Ala/Ala groups could partly explain the resultant decrease in antilipolytic effect of insulin in these two groups when compared to Pro/Pro. In addition, post-OGTT a decreased gene expression of the lipolytic enzyme HSL from fasting levels in Pro/Pro could explain the observed higher antilipolytic effect of insulin. Although a similar decrease in *HSL* gene expression was observed in Pro/Ala group, an antilipolytic effect of insulin could have been counter-regulated due to the presence of both alleles. A previous study showed a significant decrease in HSL activity 30 minutes after a meal in Pro12Ala carriers.[27] In our study, *HSL* expression on fasting in Ala/Ala group was significantly lower than two other groups, which also did not change post-OGTT like in the other two groups. This could partly explain a lower antilipolytic effect in Ala/Ala post-OGTT. Nevertheless, the HSL activity is mainly regulated at the phosphorylation level and we acknowledge that the change in gene expression of *HSL* alone is not sufficed to explain our lipolysis findings.[28] Moreover, the number of subjects in each group is limited and therefore the results should be interpreted with caution.

Adipocytes from lean subjects are smaller and display higher insulin sensitivity compared to obese.[29] Prior studies have shown a positive association between Pro12Ala and BMI . However, this association seems to be dependent on the degree of adiposity, as lean carriers of Ala variant had no difference in their mean BMI
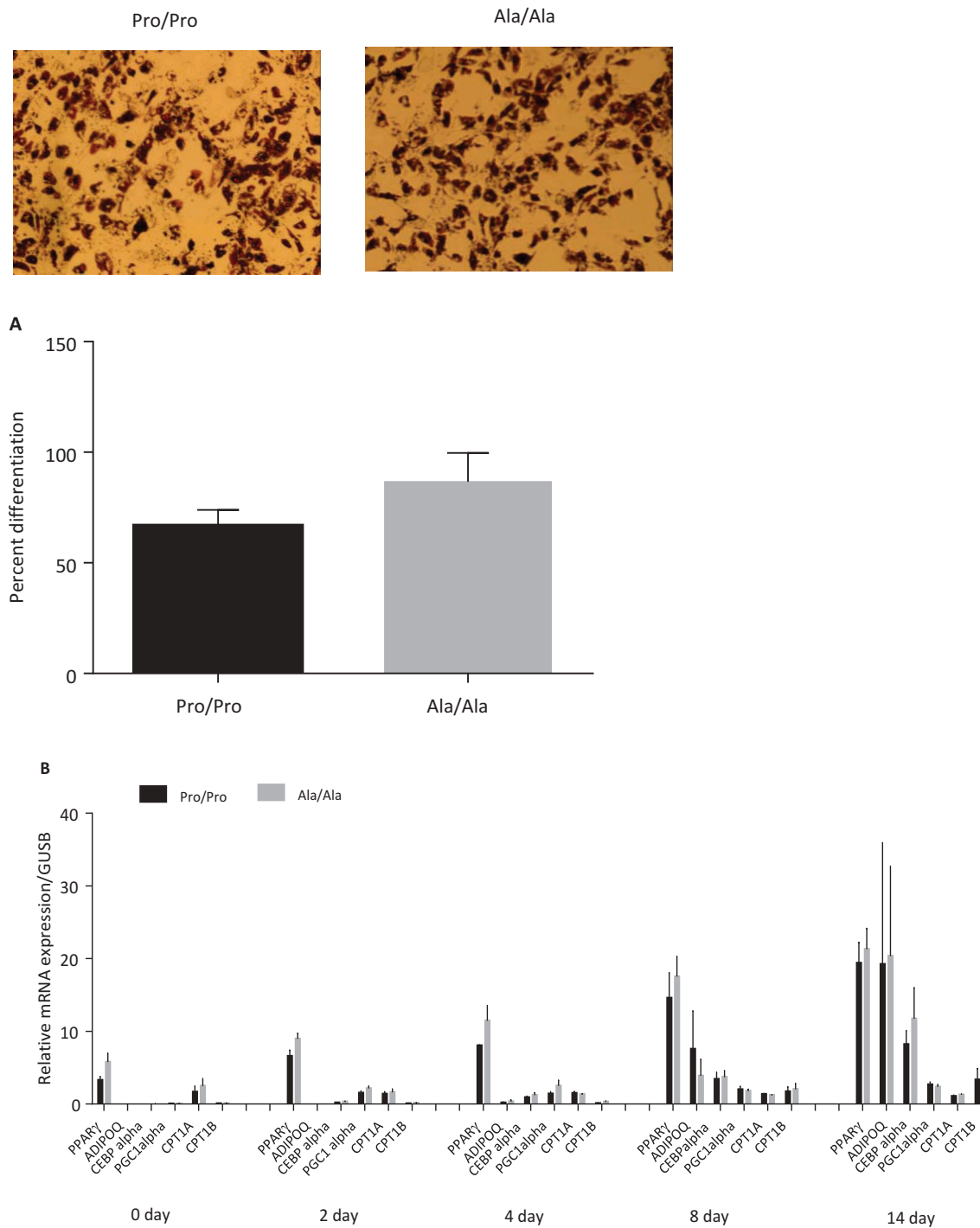
Figure 5. Effect of PPARγ Pro12Ala on preadipocytes differentiation. Preadipocytes from Pro/Pro and Ala/Ala group were differentiated as described in 'Methods'. The degree and rate of differentiation were assessed by measuring the amount of lipid accumulated at the end of differentiation and expression of different adipogenic markers at different time points during differentiation, respectively. (A) A graph showing the percent of differentiated adipocytes together with a representative image of lipid accumulation in differentiated adipocytes on the 14th day of differentiation between Pro/Pro and Ala/Ala group (6 and 7 Pro/Pro and Ala/Ala, respectively). (B) Expression of adipogenic markers at confluence (day 0), 2, 4, 8 and 14th day of differentiation (n = 6 Pro/Pro, 7 Ala/Ala). *GUSB* was used as an endogenous control. All data are presented as mean ± SEM.

ADIPOQ: adiponectin, *CEBP alpha*: CAAT enhancer binding protein alpha, *CPT1A* and *CPT1B*: Carnitine O-Palmitoyltransferase 1 alpha and 2 beta, *FAS*: fatty acid synthase, *PPARγ*: peroxisome proliferator-activated receptor gamma, *PPARGC1 alpha*: PPARγ coactivator 1 alpha. Relative mRNA expression is shown as for *ADIPOQ*: $2^{-DCT} \times 10^{-3}$ and *CEBP alpha*: $2^{-DCT} \times 10^{-1}$.

when compared to wild-type whereas, in obese subjects, Ala variant was positively associated BMI.[30] In our study cohort, neither BMI nor adipocyte size was different between genotype groups, which could in part explain the non-significant difference in metabolic assays.

Activation of PPARγ triggers differentiation of pre-adipocytes which lead to the formation of smaller and more insulin sensitive adipocytes.[31] We asked whether Pro12Ala mediates insulin sensitization by increasing subcutaneous adipocyte differentiation. However, we observed no significant difference in adipogenic potential of preadipocytes from Pro/Pro and Ala/Ala homozygotes. Previous studies in 3T3-L1 cells and in primary preadipocytes obtained from Pro12Ala knock-in mice show a decreased adipogenesis with Ala variant.[23,32] Although these studies are in concordance with the reduced transcriptional activity of Ala variant,[33] discrepancies between our findings could only be explained by the use of a different experimental model. Similar to our findings, a study in human SGBS cell line showed the non-significant effect on differentiation in Pro12Ala compared to wild-type.[34]

To the best of our knowledge, this is one of the few studies that aimed to understand the cellular mechanisms underlying the protective effect of PPARγ2 Pro12Ala polymorphism directly in human AT. However, our study has limitations. The gene expression analysis using PCR is often influenced by the choice of housekeeping genes and therefore we acknowledge that if genes other than 18S rRNA or GUSB were used, this might possibly modify the results slightly. In addition, the number of subjects included was small due to a limited number of subjects to invite, in particular within the Ala/Ala group, and the participation rate was reduced by the extensive investigation.[35] This decreased the probability of finding statistically significant differences, especially when studying a common variant like Pro12Ala with an effect size of a modest magnitude. However, based on power calculations, a comparison of two groups with the current sample sizes allowed 80% power to detect a 20% difference in insulin action on adipocyte lipolysis as well as on glucose uptake. Such a difference with respect to lipolysis could have been expected between the Pro/Pro and Ala/Ala (or even Pro/Ala) according to some previous in vivo studies[6,27] and between insulin-resistant individuals and control subjects.[36] Reductions of that magnitude are also found with respect to glucose uptake in common insulin-resistant conditions, such as T2D or obesity.[37] This may suggest that protective effects of Ala variants may be mediated by other mechanisms in AT such as inflammation, mitochondrial function or possibly through signaling, e.g. via

adipose factors such as adipokines, to other tissues besides subcutaneous AT, such as muscle and liver and also visceral AT where the metabolic effects may occur. In addition, gene-gene interaction, gene-environmental factors that were not controlled in our study could potentially influence the effect of gene variant on phenotype. Future investigations using estimates of polygenic risk score[38] or other genetic analysis tools should be undertaken to identify any confounding risk genes that could be different between different genotype groups and could potentially mask Pro12Ala effects.

In summary, we performed phenotyping of subcutaneous AT obtained from heterozygous and homozygous carriers as well as non-carriers of the PPARγ2 Pro12Ala polymorphism. No major differences were observed in the expression of PPARγ target genes in AT, insulin action on lipolysis and glucose uptake of adipocytes and adipogenesis. Potential explanations for these findings could be a too small sample size in relation to the modest effects of this common variant. Alternatively, other mechanisms in adipose tissue or effects in other tissues such as muscle or liver, including visceral AT, might contribute to the protective effects of the Pro12Ala variant. This should be addressed in future studies.

Subjects, materials and methods

Subjects

The GBR approach was used as described previously to recruit subjects for the present study.[35] In brief, 40 age and sex-matched Caucasians were recruited of which 12, 15 and 13 were Pro homozygotes (Pro/Pro, 4M/8F, mean age 64 ± SD 9y, BMI 26.8 ± SD 3.3 kg/m^2), Pro/Ala heterozygotes (Pro/Ala, 6M/9F, 63 ± SD 9y, BMI 24.3 ± SD 3.2 kg/m^2), and Ala homozygotes (Ala/Ala, 4M/9F, 64 ± SD 8y, BMI 26.6 ± SD 3.6 kg/m^2), respectively. Subjects arrived in the morning after an overnight fast (about 10 to 12 h) at the diabetes outpatient clinic at Uppsala University Hospital, Uppsala, Sweden. Anthropometric measurements were done and fasting blood samples were taken to measure the levels of plasma glucose, insulin, and lipids at the Department of Clinical Chemistry, Uppsala University Hospital. The anthropometric and clinical characteristics of subjects have been reported previously.[35] The inclusion and exclusion criteria for the present study have been reported elsewhere.[35] Briefly, subjects with diabetes and other endocrine disorder, cancer, and other major illness, as well as ongoing medication, including beta-adrenergic blockers, systemic glucocorticoids or immune-modulating therapies were

excluded from the study. A written informed consent was obtained from all study subjects and the study approval was sought from the Regional Ethics Review Board in Uppsala. All study subjects, physicians, nurses, and researchers, except the database manager, involved in the study remained blinded to genotypes until all 40 subjects had completed investigation.

Adipose tissue biopsy and isolation of adipocytes

Subcutaneous fat on fasting and after 3 h oral glucose tolerance test (OGTT) was obtained by needle aspiration after administration of local anesthetic, lidocaine (Xylocain; AstraZeneca, Sweden) from the lower part of the abdomen. Tissue was rinsed with a sterile saline solution and part of it was snap frozen within 2–3 minutes after biopsy and thereafter stored at −80° C for analysis of gene expression. The other part collected into a tube containing Hank's medium (Invitrogen Corporation, Paisley, UK) comprising 5.6 mM glucose, 4% bovine serum albumin (BSA, Sigma, St Louis, Missouri, USA), 150 nM adenosine (Sigma) maintained at 37°C and used to perform metabolic assays. AT was first digested with collagenase (Roche, Indianapolis, USA) in Hank's medium in a shaking water bath for 1 hour. Isolated adipocytes were then filtered through a 250 μm pore size nylon mesh and used to perform glucose uptake, lipolysis, and cell size measurement.

Glucose uptake

Glucose uptake and lipolysis in adipocytes were performed as described elsewhere.[39,40] In brief, isolated adipocytes were washed three times with a 5-minute interval in glucose-free Krebs-Ringer media (KRH) added with 4% BSA, 150 nM adenosine and pH 7.4. Adipocytes were then diluted at 1:10 in KRH media and incubated at 37°C with or without insulin (25 and 1000 μU/ml, Actrapid, Novo Nordisk, Bagsvaerd, Denmark) for 15 minutes, followed by an additional incubation for 45 minutes with D-[U-^{14}C] glucose (0.26 mCi/L, 0.86 μM, Perkin Elmer, Boston, MA, 161 USA). The cell suspension was transferred into pre-chilled vials to stop the reaction and cells were separated from the medium by centrifugation through silicon oil (SERVA Electrophoresis GmbH, Heidelberg, Germany). Radioactivity associated with cells was then determined using scintillation counter. Cellular glucose uptake was calculated using the following formula: Cellular clearance of medium glucose = (cell associated radioactivity x volume)/(radioactivity of medium x cell number x time). The rate of transmembrane glucose transport was mainly taken as an index of glucose uptake. Adipocyte size and cell number were determined. Glucose uptake was normalized per cell number for each experimental condition and presented as relative to basal which was set to 1. All experiments were performed in triplicates.

Lipolysis

Isolated adipocytes were washed three times with a 5-minute interval in Hank's medium (added with 5.6 mM glucose, 4% BSA, 150 nM adenosine and pH 7.4) and were suspended at 3–5% lipocrit in vials and incubated in a gently shaking water bath at 37°C for 2 hours to measure basal lipolysis. In addition, to measure the effect of the Pro12Ala polymorphism on stimulated lipolysis and on the antilipolytic effect of insulin, adipocytes were also incubated with isoproterenol (0.5 μM, Sigma) or isoproterenol (0.5 μM) and insulin (0.1–100 μU/ml), respectively. All experiments were performed in triplicates. After 2 hours, the reaction was stopped by transferring the vials on ice. Glycerol released into the medium was measured by the quantitative enzymatic reaction using Free Glycerol Reagent (Sigma) and absorbance was read at 540 nm using a microplate reader (Infinite®200, Tecan, and Männedorf, Switzerland). Cellular lipids were extracted[41] and cell number was measured as previously described.[42] For each experimental condition, glycerol released into the medium was taken as lipolysis index and normalized per cell number (nmol/10^5/cells/h) and presented as glycerol released relative to maximal (isoproterenol-stimulated lipolysis) which was set to 1.

Gene expression analysis

Total RNA was extracted from AT using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) and reverse transcribed using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). The protocol was carried out as per manufacturer guidelines. The concentration and purity of total RNA were measured with the Nanodrop (Thermo Scientific) to ensure the reliability of subsequent RNA processing and mRNA assessments. The 260/280 and 260/230 ratios for concentration and purity were in the acceptable range, therefore, none of the RNA samples were excluded from further analysis. TaqMan gene expression assays (Thermo Fisher) were used to study the expression levels of different transcript. The gene expression was detected using QuantStudio 3 sequence detection system (Applied Biosystem). The data were calculated using a relative

standard curve method or $2^{-\text{delta Ct}}$ as specified wherever necessary. The results are plotted as relative quantification using 18S ribosomal RNA (18S rRNA) or glucuronidase beta (GUSB) as an endogenous control. Both are standard housekeeping genes and have been validated in studies of human adipose tissue.[43,44] For differentiation assays, we exclusively used GUSB because of high variability in the expression of 18S rRNA in response to treatment with differentiation cocktail. In addition, GUSB has also been shown to be stably expressed during preadipocyte differentiation.[45] All samples were run in duplicates. The details of the TaqMan assay used are given in the Supplementary Table 1.

Adipogenesis

Isolation and culture of human stromal vascular fraction (SVF)

After AT digestion with collagenase, the SVF containing preadipocytes was separated from mature adipocytes into a Falcon tube. The SVF was centrifuged at 1200 RPM for 3 minutes and the pellet was cultured in preadipocytes medium (DMEM-F12 containing 10% foetal calf serum (FCS) (Invitrogen), 100 units/ml penicillin and 100 g/ml streptomycin (PEST, Life Technology) and 17 ng/ml basic fibroblast growth factor (bFGF) (Sigma)) at 37°C. Media was replaced after every two days. After the cells reached about 70% confluence, they were trypsinized and stored in DMEM-F12 with 20% FCS, 10% dimethyl sulfoxide (DMSO) at −150°C until further analysis (Passage 0).

Differentiation of preadipocytes

Preadipocytes from passage 0 were thawed at 37°C and expanded into a T-75 flask using preadipocytes medium (passage 1). Upon reaching 70% confluence the cells were trypsinized and seeded again into a 12 well plate at density 15,000 cells/cm^2 (passage 2) using preadipocytes media. After the cells reached 100% confluence, the differentiation was induced by adding a differentiation cocktail DMEM-F12, 1% PEST, 100 nM insulin, 17 µM pantothenate (Sigma), 33 µM biotin (Sigma), 1 µM dexamethasone (Sigma), 1 µM rosiglitazone (Sigma), 250 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 10 µg/ml transferrin (Sigma), 2 nM triiodothyronine (T3, Sigma) for 5 days. The differentiation continued using a maintenance media (composition is same as that of differentiation cocktail except for IBMX) until 14 days. The medium was replenished every 2 days. The rate of differentiation was assessed by measuring the expression levels of differentiation markers in cells collected on confluence and 2, 4, 8 and 14 days post induction. The GUSB was used as a

housekeeping gene for differentiation experiment due to the observed variation in the expression of another housekeeping gene, 18S rRNA, in response to treatment with differentiation cocktail.

On the 14th day of differentiation, media was removed and cells were washed with PBS and fixed with 4% formaldehyde (Histolab, Gothenburg, Sweden) for 30 minutes at room temperature. The cells were again washed two times with PBS and lipid was stained with 0.5% red oil dissolved in iso-propanol (Sigma) for 30 minutes. The cells were washed two times with ddH$_2$O and nuclei were counterstained using 20 µM 2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI, Thermo Fisher) for 7 minutes.

The images of differentiated cells were acquired from three different randomly chosen areas under a microscope (Evos, Invitrogen). The total area covered by red oil (lipid stains) and a number of nuclei stained with DAPI were measured. The percent differentiation was calculated using the following formula: percent differentiation = area covered by lipid/number of nuclei * 100.

Statistical analysis

IBM SPSS statistical software version 22 was used and all data are presented as mean± SEM unless otherwise indicated. All data were first checked for the distribution of normality using Shapiro-Wilk test and visual inspection of a histogram. Comparisons of glucose uptake, lipolysis, gene expression and adipocyte size between Pro/Pro, Pro/Ala, and Ala/Ala groups were done using one-way ANOVA or Kruskal-Wallis test depending upon normally and non-normally distributed data, respectively. Independent sample t-test or Mann-Whitney U test, respectively, was then used for post-hoc testing. The difference between fasting and post-OGTT within individuals of the same group was analyzed using the Wilcoxon test. A p-value < 0.05 was considered statistically significant. Based on previous adipocyte studies and standard deviations of insulin-inhibited lipolysis[46] and insulin-stimulated glucose uptake,[47] the study had at least 80% power to detect 20% differences in those effects of insulin between different genotype groups (n at least 12 Pro12Pro and 13 Ala12Ala).

Acknowledgments

Disclosure statement

Funding

References

1. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, Evans RM. PPARgamma signaling and metabolism: the good, the bad and the future. Nat Med. 2013;19(5):557–566.
2. Saltiel AR, Olefsky JM. Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes. 1996;45(12):1661–1669.
3. Elbrecht A, Chen Y, Cullinan CA, Hayes N, Leibowitz MD, Moller DE, Berger J. Molecular cloning, expression and characterization of human peroxisome proliferator activated receptors gamma 1 and gamma 2. Biochem Biophys Res Commun. 1996;224(2):431–437.
4. Werman A, Hollenberg A, Solanes G, Bjorbaek C, Vidal-Puig AJ, Flier JS. Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and −2 isoforms and influence of insulin. J Biol Chem. 1997;272(32):20230–20235.
5. Yen CJ, Beamer BA, Negri C, Silver K, Brown KA, Yarnall DP, Burns DK, Roth J, Shuldiner AR. Molecular scanning of the human peroxisome proliferator activated receptor gamma (hPPAR gamma) gene in diabetic Caucasians: identification of a Pro12Ala PPAR gamma 2 missense mutation. Biochem Biophys Res Commun. 1997;241(2):270–274.
6. Stumvoll M, Wahl HG, Loblein K, Becker R, Machicao F, Jacob S, Häring H. Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-gamma2 gene is associated with increased antilipolytic insulin sensitivity. Diabetes. 2001;50(4):876–881.
7. Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J, Lane CR, Schaffner SF, Bolk S, Brewer C, et al. The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet. 2000;26(1):76–80.
8. Jacob S, Stumvoll M, Becker R, Koch M, Nielsen M, Löblein K, Maerker E, Volk A, Renn W, Balletshofer B, et al. The PPARgamma2 polymorphism pro12Ala is associated with better insulin sensitivity in the offspring of type 2 diabetic patients. Horm Metab Res. 2000;32(10):413–416.
9. Tonjes A, Scholz M, Loeffler M, Stumvoll M. Association of Pro12Ala polymorphism in peroxisome proliferator-activated receptor gamma with Pre-diabetic phenotypes: meta-analysis of 57 studies on nondiabetic individuals. Diabetes Care. 2006;29(11):2489–2497.
10. Barbieri M, Rizzo MR, Papa M, Acampora R, De Angelis L, Olivieri F, Marchegiani F, Franceschi C, Paolisso G. Role of interaction between variants in the PPARG and interleukin-6 genes on obesity related metabolic risk factors. Exp Gerontol. 2005;40(7):599–604.
11. Swarbrick MM, Chapman CM, McQuillan BM, Hung J, Thompson PL, Beilby JP. A Pro12Ala polymorphism in the human peroxisome proliferator-activated receptor-gamma 2 is associated with combined hyperlipidaemia in obesity. Eur J Endocrinol. 2001;144(3):277–282.
12. Iwata E, Matsuda H, Fukuda T, Fukuen S, Motomura T, Igarashi T, Yamamoto I, Azuma J. Mutations of the peroxisome proliferator-activated receptor gamma (PPAR gamma) gene in a Japanese population: the Pro12Ala mutation in PPAR gamma 2 is associated with lower concentrations of serum total and non-HDL cholesterol. Diabetologia. 2001;44(10):1354–1355.
13. Hamada T, Kotani K, Tsuzaki K, Sano Y, Murata T, Tabata M, Sato S, Sakane N. Association of Pro12Ala polymorphism in the peroxisome proliferator-activated receptor gamma2 gene with small dense low-density lipoprotein in the general population. Metabolism. 2007;56(10):1345–1349.
14. Robitaille J, Després JP, Perusse L, Vohl MC. The PPAR-gamma P12A polymorphism modulates the relationship between dietary fat intake and components of the metabolic syndrome: results from the Québec Family Study.. Clin Genet. 2003;63(2):109–116.
15. He W. PPARgamma2 polymorphism and human health. PPAR Res. 2009;2009:849538.
16. Fajas L, Auboeuf D, Raspé E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, et al. The organization, promoter analysis, and expression of the human PPARgamma gene. J Biol Chem. 1997;272(30):18779–18789.
17. Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. J Clin Invest. 1996;97(11):2553–2561.
18. Knouff C, Auwerx J. Peroxisome proliferator-activated receptor-gamma calls for activation in moderation: lessons from genetics and pharmacology. Endocr Rev. 2004;25(6):899–918.
19. Fredriksson J, Carlsson E, Orho-Melander M, Groop L, Ridderstråle M. A polymorphism in the adiponectin gene influences adiponectin expression levels in visceral fat in obese subjects. Int J Obes (Lond). 2006;30(2):226–232.
20. Pihlajamäki J, Schwab U, Kaminska D, Ågren J, Kuusisto J, Kolehmainen M, Paananen J, Laakso M, Uusitupa M. Dietary polyunsaturated fatty acids and the Pro12Ala polymorphisms of PPARG regulate serum lipids through divergent pathways: a randomized crossover clinical trial. Genes Nutr. 2015;10(6):43.

21. Boden G, Cheung P, Mozzoli M, Fried SK. Effect of thiazolidinediones on glucose and fatty acid metabolism in patients with type 2 diabetes. Metabolism. 2003;52 (6):753–759.

22. Virtanen KA, Hällsten K, Parkkola R, Janatuinen T, Lönnqvist F, Viljanen T, Rönnemaa T, Knuuti J, Huupponen R, Lönnroth P, et al. Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose uptake in type 2 diabetic subjects. Diabetes. 2003;52(2):283–290.

23. Heikkinen S, Argmann C, Feige JN, Koutnikova H, Champy M-F, Dali-Youcef N, Schadt EE, Laakso M, Auwerx J. The Pro12Ala PPARgamma2 variant determines metabolism at the gene-environment interface. Cell Metab. 2009;9(1):88–98.

24. Vänttinen M, Nuutila P, Pihlajamäki J, Hällsten K, Virtanen KA, Lautamäki R, Peltoniemi P, Kemppainen J, Takala T, Viljanen APM, et al. The effect of the Ala12 allele of the peroxisome proliferator-activated receptor-gamma2 gene on skeletal muscle glucose uptake depends on obesity: a positron emission tomography study. J Clin Endocrinol Metab. 2005;90 (7):4249–4254.

25. Honka MJ, Vanttinen M, Iozzo P, Virtanen KA, Lautamäki R, Hällsten K, Borra RJH, Takala T, Viljanen APM, Kemppainen J, et al. The Pro12Ala polymorphism of the PPARgamma2 gene is associated with hepatic glucose uptake during hyperinsulinemia in subjects with type 2 diabetes mellitus. Metabolism. 2009;58(4):541–546.

26. Medina-Gomez G, Virtue S, Lelliott C, Boiani R, Campbell M, Christodoulides C, Perrin C, Jimenez-Linan M, Blount M, Dixon J, et al. The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform. Diabetes. 2005;54 (6):1706–1716.

27. Tan GD, Neville MJ, Liverani E, Humphreys SM, Currie JM, Dennis L, Fielding BA, Karpe F. The in vivo effects of the Pro12Ala PPARgamma2 polymorphism on adipose tissue NEFA metabolism: the first use of the Oxford Biobank. Diabetologia. 2006;49(1):158–168.

28. Large V, Arner P, Reynisdottir S, Grober J, Van Harmelen V, Holm C, Langin D. Hormone-sensitive lipase expression and activity in relation to lipolysis in human fat cells. J Lipid Res. 1998;39(8):1688–1695.

29. Laforest S, Labrecque J, Michaud A, Cianflone K, Tchernof A. Adipocyte size as a determinant of metabolic disease and adipose tissue dysfunction. Crit Rev Clin Lab Sci. 2015;52(6):301–313.

30. Ek J, Urhammer SA, Sorensen TI, Andersen T, Auwerx J, Pedersen O. Homozygosity of the Pro12Ala variant of the peroxisome proliferation-activated receptor-gamma2 (PPAR-gamma2): divergent modulating effects on body mass index in obese and lean Caucasian men. Diabetologia. 1999;42(7):892–895.

31. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, et al. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J Clin Invest. 1998;101(6):1354–1361.

32. Masugi J, Tamori Y, Mori H, Koike T, Kasuga M. Inhibitory effect of a proline-to-alanine substitution at codon 12 of peroxisome proliferator-activated receptor-gamma 2 on thiazolidinedione-induced adipogenesis. Biochem Biophys Res Commun. 2000;268(1):178–182.

33. Deeb SS, Fajas L, Nemoto M, Pihlajamäki J, Mykkänen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J. A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nat Genet. 1998;20(3):284–287.

34. Majithia AR, Flannick J, Shahinian P, Guo M, Bray M-A, Fontanillas P, Gabriel SB, Rosen ED, Altshuler D. Rare variants in PPARG with decreased activity in adipocyte differentiation are associated with increased risk of type 2 diabetes. Proc Natl Acad Sci U S A. 2014;111(36):13127–13132.

35. Kamble PG, Gustafsson S, Pereira MJ, Lundkvist P, Cook N, Lind L, Franks PW, Fall T, Eriksson JW, Ingelsson E. Genotype-based recall to study metabolic effects of genetic variation: a pilot study of PPARG Pro12Ala carriers. Ups J Med Sci. 2018;122:1–9.

36. Armstrong MJ, Hazlehurst JM, Hull D, Guo K, Borrows S, Yu J, Gough SC, Newsome PN, Tomlinson JW. Abdominal subcutaneous adipose tissue insulin resistance and lipolysis in patients with non-alcoholic steatohepatitis. Diabetes Obes Metab. 2014;16(7):651–660.

37. Pereira MJ, Skrtic S, Katsogiannos P, Abrahamsson N, Sidibeh CO, Dahgam S, Månsson M, Risérus U, Kullberg J, Eriksson JW. Impaired adipose tissue lipid storage, but not altered lipolysis, contributes to elevated levels of NEFA in type 2 diabetes. Degree of hyperglycemia and adiposity are important factors. Metabolism. 2016;65(12):1768–1780.

38. Lall K, Magi R, Morris A, Metspalu A, Fischer K. Personalized risk prediction for type 2 diabetes: the potential of genetic risk scores. Genet Med. 2017;19 (3):322–329.

39. Yu ZW, Jansson PA, Posner BI, Smith U, Eriksson JW. Peroxovanadate and insulin action in adipocytes from NIDDM patients. Evidence against a primary defect in tyrosine phosphorylation. Diabetologia. 1997;40 (10):1197–1203.

40. Sidibeh CO, Pereira MJ, Lau Borjesson J, Kamble PG, Skrtic S, Katsogiannos P, Sundbom M, Svensson MK, Eriksson JW. Role of cannabinoid receptor 1 in human adipose tissue for lipolysis regulation and insulin resistance. Endocrine. 2017;55(3):839–852.

41. Dole VP. The fatty acid pool in adipose tissue. J Biol Chem. 1961;236:3121–3124.

42. Lundgren M, Svensson M, Lindmark S, Renström F, Ruge T, Eriksson JW. Fat cell enlargement is an independent marker of insulin resistance and 'hyperleptinaemia'. Diabetologia. 2007;50(3):625–633.

43. Fonseca A, Carvalho E, Eriksson JW, Pereira MJ. Calcineurin is an important factor involved in glucose uptake in human adipocytes. Mol Cell Biochem. 2018;445:157–168.

44. Viguerie N, Montastier E, Maoret JJ, Roussel B, Combes M, Valle C, Villa-Vialaneix N, Iacovoni JS,

Martinez JA, Holst C, et al. Determinants of human adipose tissue gene expression: impact of diet, sex, metabolic status, and cis genetic regulation. PLoS Genet. 2012;8(9):e1002959.

45. Fink T, Lund P, Pilgaard L, Rasmussen J, Duroux M, Zachar V. Instability of standard PCR reference genes in adipose-derived stem cells during propagation, differentiation and hypoxic exposure. BMC Mol Biol. 2008;9:98.

46. Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK, Eriksson JW. The immunosuppressive agents rapamycin, cyclosporin A and tacrolimus increase lipolysis, inhibit lipid storage and alter expression of genes involved in lipid metabolism in human adipose tissue. Mol Cell Endocrinol. 2013;365(2):260–269.

47. Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK, Eriksson JW. mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes. Mol Cell Endocrinol. 2012;355(1):96–105.