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The in vivo effects of the Pro12Ala PPAR γ 2 polymorphism on adipose tissue NEFA metabolism: the first use of the Oxford Biobank

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Abstract *Aims/hypothesis:* To investigate the phenotypic effects of common polymorphisms on adipose tissue metabolism and cardiovascular risk factors, we set out to establish a biobank with the unique feature of allowing a prospective recruit-by-genotype approach. The first use of this biobank investigates the effects of the peroxisome proliferator-activated receptor- γ (PPAR γ) Pro12Ala polymorphism on integrative tissue-specific physiology. We hypothesised that Ala12 allele carriers demonstrate greater adipose tissue metabolic flexibility and insulin sensitivity. *Materials and methods:* From a comprehensive population register, subjects were recruited into a biobank, which was genotyped for the Pro12Ala polymorphism. Twelve healthy male Ala12 carriers and 12 matched Pro12 homozygotes underwent detailed physiological phenotyping using stable isotope techniques, and measurements of blood flow and arteriovenous differences in adipose tissue and muscle in response to a mixed meal containing [1,1,1-¹³C]tripalmitin. *Results:* Of 6,148 invited subjects, 1,072 were suitable for inclusion in the biobank. Among Pro12 homozygotes, insulin sensitivity correlated with HDL-cholesterol concentrations, and inversely correlated with blood pressure, apolipoprotein B, triglyceride and total cholesterol concentrations. Ala12 carriers showed no such correlations. In the meal study, Ala12 carriers had lower plasma NEFA concentrations, higher adipose tissue and muscle blood flow, and greater insulin-mediated postprandial hormone-sensitive lipase suppression along with greater insulin sensitivity than Pro12 homozygotes. *Conclusions/interpretation:* This study shows that a recruit-by-genotype approach is feasible and describes the biobank's first application, providing tissue-specific physiological find-

ings consistent with the epidemiological observation that the PPAR γ Ala12 allele protects against the development of type 2 diabetes.

Keywords Genetics · Insulin sensitivity · Obesity · Peroxisome proliferator-activated receptors · PPAR γ 2 · Pro12Ala polymorphism · Single nucleotide polymorphism · Type 2 diabetes

Abbreviations apoB: apolipoprotein B · HOMA-IR: homeostasis model assessment for insulin resistance · HSL: hormone-sensitive lipase · OBB: Oxford Biobank · PPAR γ : peroxisome proliferator-activated receptor- γ

Introduction

The gene for peroxisome proliferator-activated receptor- γ , *PPARG*, plays a key role in insulin resistance and type 2 diabetes, as well as adipocyte differentiation and lipid metabolism [1]. The Pro12Ala polymorphism of the PPAR γ 2 isoform has an Ala12 allele frequency of around 0.12 in Caucasians [2]. Epidemiological studies have shown an association of the Pro12 allele with an increased risk of developing type 2 diabetes, with a population-attributable risk of 25% [3, 4]. In vivo in humans, Ala12 allele carriers are more insulin-sensitive than Pro12 homozygotes [5–9]. Although PPAR γ activation by thiazolidinediones leads to insulin sensitisation, in vitro studies of the polymorphism have shown that the substitution of Ala for Pro results in reduced transcriptional activity of *PPARG* [5, 10].

The role of the Pro12Ala polymorphism in the development of obesity is less clear. A meta-analysis suggested that Ala12 carriers were more likely to have a high BMI than Pro12 homozygotes, an effect only identifiable among subjects with a BMI greater than 27 kg/m² [11]. Thus, Ala12 carriers appear to be heavier than Pro12 homozygotes, yet have a lower risk of developing type 2 diabetes. However, the mechanism underlying this apparent paradox is unclear.

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Subjects with type 2 diabetes treated with the PPAR γ agonist rosiglitazone exhibit a similar unexpected dissociation between weight and insulin sensitivity: increasing weight is associated with an increase in insulin sensitivity. However, some weight gain may be due to fluid retention, as well as increasing fat mass. The exact mechanisms of insulin sensitisation are unclear, although we have previously documented changes in fatty acid metabolism in subjects treated with rosiglitazone [12].

We hypothesised that Ala12 carriers are more efficient in sequestering fatty acids in adipose tissue, and demonstrate greater metabolic flexibility in the storage and mobilisation of NEFAs in the postprandial period. Such a mechanism would increase insulin sensitivity and decrease the likelihood of developing type 2 diabetes, and would also be consistent with the increased adipose tissue mass observed in Ala12 carriers.

Such a complex genotype–phenotype relationship cannot be identified from stored fasting plasma. A useful approach could be to start by identifying carriers of certain genotypes from large cohort studies (i.e. genotyping many individuals). From such a large database of volunteers, closely matched subjects, differing as far as possible only in their genotype of interest, could be prospectively recruited into a study for detailed phenotyping. This recruit-by-genotype approach would allow the study of the influences of common genetic variants on metabolism. Prospective recruitment allows close baseline matching, along with comprehensive characterisation of the phenotype of interest, and adequately powered studies. Such biobanks have been set up to investigate other complex intermediate phenotypes [13, 14], but never before for insulin resistance and type 2 diabetes.

We therefore set out to establish a biobank, known as the Oxford Biobank (OBB), to allow the prospective investigation of genotype–phenotype relationships in adipose tissue and human metabolism. The OBB included DNA, anthropometric and biochemical data of healthy subjects taken at random from the general population. Recruited subjects would be willing to participate in detailed metabolic phenotyping in order to investigate insulin resistance and type 2 diabetes.

The possible functional impact of the PPAR γ 2 Pro12Ala polymorphism, along with its high prevalence in the Caucasian population, made it a promising candidate for detailed tissue-specific metabolic phenotyping using the recruit-by-genotype approach. As *PPARG* is highly expressed in adipose tissue [15] and as subcutaneous abdominal adipose tissue is the major source of circulating NEFA in humans [16], we chose to phenotype subcutaneous abdominal adipose tissue function in Pro12 homozygotes and Ala12 allele carriers, with particular emphasis on fatty acid and triglyceride metabolism *in vivo*, in order to test our hypothesis that the Ala12 allele carriers would handle NEFA metabolism more efficiently in adipose tissue and skeletal muscle. These inferences have already been drawn, but have never been tested directly at the level of adipose tissue.

Subjects and methods

Oxford Biobank

Using the comprehensive population register maintained by the UK National Health Service, an age-stratified random sample of 30- to 50-year-old men and women from Oxfordshire (total population 615,200 [17]) was selected. Exclusion criteria included mental or physical ill health, alcohol- or drug-related problems, and abnormal biochemical data as determined by history, examination, routine blood tests and information obtained from each subject's primary care physician. Subjects attended a screening visit at the Clinical Research Unit, where blood tests were performed, including plasma glucose, insulin and triglyceride analysis. Basic anthropometric data were recorded, including weight, height and skinfold measurements. DNA was stored from the visit and consent obtained to allow subsequent genotyping for current and future genes of potential importance in adipose tissue metabolism. Samples were genotyped for the Pro12Ala polymorphism by PCR sequence-specific amplification, as previously described [18], using the allele-specific primers: 5'-TGAAG GAATCGCTTTCTGG-3' and 5'-TGAAGGAATCGCTT TCTGC-3' and the consensus primer 5'-GTCACCGGC GAGACAG-3' to generate an allele-specific product of 357 nucleotides. The products were then visualised on a 1% agarose gel containing ethidium bromide.

Pro12Ala meal studies

Subjects

From the OBB register, 24 healthy non-diabetic subjects were recruited by genotype. Twelve Ala12 heterozygotes and 12 matched Pro12 homozygotes were selected. No Ala12 homozygotes were used because no suitable subjects were available to participate. As smoking can affect insulin sensitivity, only non-smoking subjects were recruited. The study was approved by the Oxfordshire Research Ethics Committee and all subjects gave their written informed consent.

To assess the interaction of weight and genotype, we deliberately set out to recruit some subjects who were of normal weight and some subjects who were overweight into both genotype groups. Of the 24 subjects, 12 were overweight (six Pro12 homozygotes and six Ala12 carriers) and 12 were of normal weight (six Pro12 homozygotes and six Ala12 carriers). Overweight subjects had a BMI greater than 28.5 kg/m² and were in the highest quartile of subjects in the OBB by weight. Normal-weight subjects had a BMI less than 25.5 kg/m² and were in the lower half of subjects in the OBB by weight.

The 24 selected subjects attended the Clinical Research Unit for a full-day metabolic investigation. Vigorous exercise and alcohol were avoided for 24 h and food for

10 h before the study. During the study day, arteriovenous differences in metabolites across adipose tissue and skeletal muscle were measured, along with tissue blood flow. Body fat was measured by bioimpedance using a Bodystat 500 (Bodystat, Douglas, UK).

Metabolic investigation protocol

Recording arteriovenous differences across adipose tissue and skeletal muscle enabled the measurement of differences between the composition of venous blood draining a particular tissue and arterialised blood, thus reflecting that tissue's metabolic activity. Inclusion of tissue blood flow into the calculations allows quantification of net metabolic activity. On the day of the study, serial blood samples were taken in the fasting state and for 6 h after metabolic provocation with a standardised mixed meal, a technique we have used elsewhere [12].

Arterialised blood was obtained from a vein draining a heated hand. Venous blood from muscle was taken retrogradely from a vein draining the deep structures of the forearm. Venous blood from adipose tissue was obtained from the superficial epigastric vein, as described previously [19]. This vein drains subcutaneous abdominal adipose tissue with negligible contribution from other tissues [20]. Oxygen saturation and ultrasonography were used to confirm adequate siting of the cannulae. Blood samples were taken simultaneously from all three sites. To prevent contamination of the blood from the forearm vein with blood from the hand, a wrist cuff was inflated to 200 mmHg for 3 min before taking samples. Two sets of baseline blood samples were taken 30 min apart. Patients then consumed a meal of a fat emulsion, skimmed milk and Rice Krispies (Kellogg Company, Manchester, UK) containing 40 g fat and 40 g carbohydrate and 600 mg [1,1,1-¹³C]tripalmitin. Further blood samples were taken for 6 h after the meal.

Plasma triglyceride, NEFA, glucose and insulin and blood glycerol concentrations were measured as described previously [21]. Adiponectin was measured using ELISA (Linco, St Charles, MO, USA).

The postprandial increase in ¹³C in the circulation was derived from the [1,1,1-¹³C]tripalmitin in the meal. NEFAs and triglycerides were extracted from fasting and postprandial arterialised plasma samples for gas chromatography and gas chromatography–combustion–isotope ratio mass spectrometry. Enrichments of [¹³C]palmitate in plasma NEFA and triglyceride fractions were measured. The results were multiplied by the concentrations of palmitate–NEFA and palmitate–triglyceride, respectively to give the concentrations of the tracer in these fractions, as previously described [21]. The rate of appearance in expired air of ¹³CO₂ from [¹³C]palmitate was measured using gas chromatography–isotope ratio mass spectrometry, providing an index of whole-body fatty acid oxidation.

Subcutaneous abdominal adipose tissue blood flow was measured by ¹³³Xe washout [22]. Forearm muscle blood flow was assessed by strain-gauge plethysmography [23].

Statistical analyses, power and calculations

Adipose tissue blood flow was calculated as described previously [22]. Arteriovenous and venoarterial differences

Table 1 Anthropometric, biochemical and haemodynamic data of subjects in the Oxford Biobank

Number of subjects	
Male	550
Female	524
Frequency of Ala12 allele	
Male (349 genotyped)	0.136
Female (420 genotyped)	0.135
Age (years)	
Male	42 (36–45)
Female	41 (35–47)
Body mass index (kg/m ²)	
Male	26.0 (23.9–28.7)
Female	24.4 (22.2–28.4)
Waist circumference (cm)	
Male	92 (85–99)
Female	78 (72–87)
Hip circumference (cm)	
Male	100 (96–104)
Female	100 (94–106)
Weight (kg)	
Male	82.6 (75.3–91.8)
Female	67.0 (60.3–76.5)
Height (m)	
Male	178 (174–183)
Female	165 (161–169)
NEFA (μmol/l)	
Male	450 (332–598)
Female	535 (393–704)
Glucose (mmol/l)	
Male	5.27 (5.01–5.56)
Female	5.00 (4.76–5.29)
Insulin (pmol/l)	
Male	58 (41–87)
Female	51 (37–75)
Triglyceride (mmol/l)	
Male	1.23 (0.88–1.69)
Female	0.90 (0.66–1.20)
Cholesterol (mmol/l)	
Male	5.50 (4.88–6.19)
Female	5.29 (4.75–6.03)
HDL-cholesterol (mmol/l)	
Male	1.22 (1.05–1.40)
Female	1.47 (1.25–1.71)
Systolic blood pressure (mmHg)	
Male	123 (116–130)
Female	112 (104–120)
Diastolic blood pressure (mmHg)	
Male	80 (74–86)
Female	74 (69–81)

Values are median (interquartile range)

in metabolite concentrations were calculated. Absolute flux was calculated as the product of the arteriovenous or venoarterial difference and adipose tissue blood flow [19]. Lipoprotein lipase rate of action in vivo was calculated from triglyceride removal across each tissue [19]. The rate of action of hormone-sensitive lipase (HSL) in adipose tissue in vivo (i.e. the rate of adipose tissue lipolysis) was calculated from the total adipose tissue glycerol release after subtraction of lipoprotein lipase rate of action [19].

The study was powered to detect a 12.5% difference in fasting NEFA output from adipose tissue, with a power of 90% at a statistical significance level of 0.05.

Data were analysed using SPSS for Windows v10 (SPSS UK, Chertsey, UK) and statistical significance was set at $p < 0.05$ for all tests. Variables are presented as median (interquartile range) unless otherwise stated. Repeated

measures ANOVA was used to identify time effects, drug effects and time by drug interactions. AUCs were compared using Wilcoxon signed rank tests.

Results

Results of Oxford Biobank

To date, 6,148 subjects have been invited to participate in the OBB register, and recruitment is ongoing; 1,835 subjects (30%) responded to the invitation letter. Of these 1,835 subjects, 1,115 (61%) attended for screening and 1,072 of them (96%) were considered suitable for inclusion in the OBB database. To date, 769 (72%) (349 male) have been genotyped for the PPAR γ 2 Pro12Ala polymorphism. Of those genotyped, 573 were homozygous for Pro12 (258 male), 184 were heterozygous (87 male) and 12 were

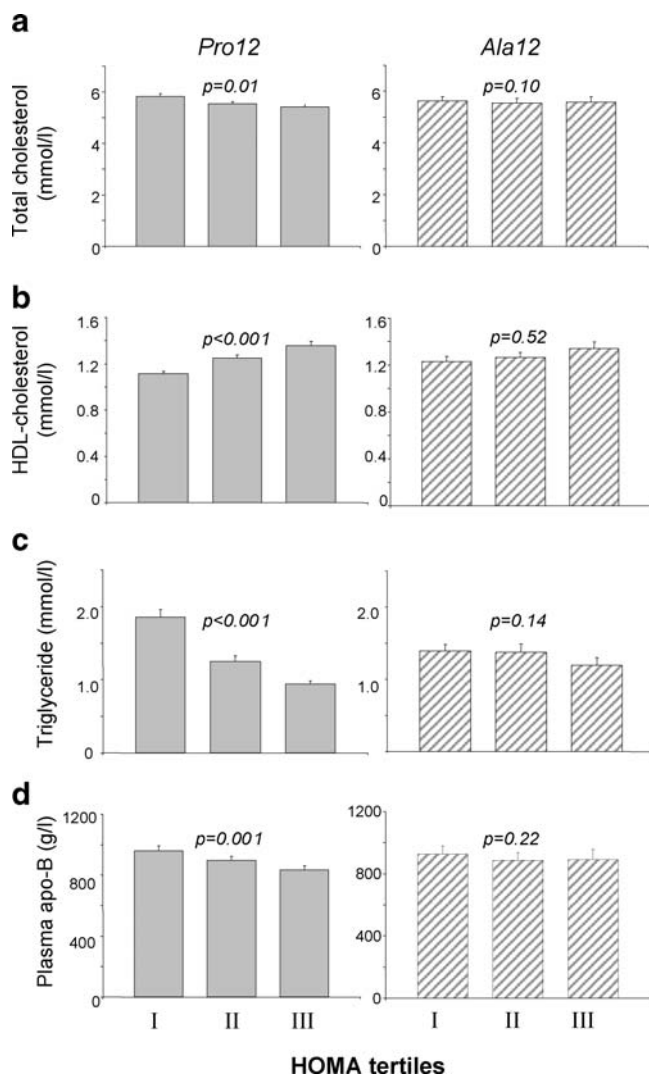


Fig. 1 The Oxford Biobank: effect of the Pro12Ala genotype on metabolic parameters according to insulin sensitivity in men. Plasma total cholesterol (a), HDL-cholesterol (b), triglyceride (c) and apoB (d) concentrations of subjects in the Oxford Biobank, divided according to genotype and tertile of insulin sensitivity (I to III in rising order of sensitivity) within the genotype. The p values are for trend of change between tertiles of insulin sensitivity

Table 2 Characteristics of subjects in the Pro12Ala meal provocation study

	p value
Number of subjects	
Pro12 homozygotes	12 men
Ala12 carriers	12 men
Age (years)	0.51
Pro12 homozygotes	47.5 (45.8–48.8)
Ala12 carriers	47.0 (42.8–50.3)
Body mass index (kg/m ²)	0.83
Pro12 homozygotes	27.7 (24.5–31.5)
Ala12 carriers	27.2 (24.7–31.3)
Fasting insulin (pmol/l)	0.38
Pro12 homozygotes	48.8 (36.0–68.8)
Ala12 carriers	46.7 (35.1–64.9)
Fasting glucose (mmol/l)	0.42
Pro12 homozygotes	5.14 (4.88–5.39)
Ala12 carriers	5.09 (4.83–5.40)
Total cholesterol (mmol/l)	0.25
Pro12 homozygotes	5.43 (4.84–6.09)
Ala12 carriers	5.44 (4.88–6.31)
Fasting triglyceride (mmol/l)	0.90
Pro12 homozygotes	0.99 (0.70–1.33)
Ala12 carriers	1.06 (0.81–1.44)
HDL-cholesterol (mmol/l)	0.77
Pro12 homozygotes	1.34 (1.15–1.57)
Ala12 carriers	1.36 (1.17–1.59)
Plasma apoB (g/l)	0.50
Pro12 homozygotes	810 (658–994)
Ala12 carriers	802 (687–1,000)
Systolic blood pressure (mmHg)	0.67
Pro12 homozygotes	116 (107–125)
Ala12 carriers	115 (108–124)
Diastolic blood pressure (mmHg)	0.42
Pro12 homozygotes	75 (70–82)
Ala12 carriers	77 (71–83)

Values are median (interquartile range)

homozygous for Ala12 (four male). Thus the Ala12 allele frequency was 0.135. The genotype frequencies of the polymorphism were in Hardy–Weinberg equilibrium ($p=0.32$). Anthropometric, biochemical and haemodynamic data of subjects recruited into the OBB are presented in Table 1.

Among men, Pro12 homozygotes showed the expected correlation between insulin resistance, assessed by homeostasis model assessment for insulin resistance (HOMA-IR), and lipid parameters (plasma triglyceride concentrations, $p<0.001$; total cholesterol concentrations, $p=0.01$; and plasma apoB concentrations, $p=0.001$). As expected, insulin resistance correlated negatively with HDL-cholesterol concentrations ($p<0.001$) among the Pro12 homozygotes. Surprisingly, insulin sensitivity among male Ala12 carriers did not correlate with any of the lipid parameters (triglycerides, $p=0.14$; total cholesterol $p=0.10$; apoB, $p=0.22$; HDL-cholesterol, $p=0.52$). These data are presented in Fig. 1, where subjects are divided into tertiles of insulin sensitivity (assessed by HOMA-IR). Among women, lipid parameters correlated with insulin sensitivity, irrespective of genotype (data not shown).

The genotype-specific relationship between insulin sensitivity and features of the metabolic syndrome extended beyond biochemical parameters. Systolic and diastolic blood pressures in males correlated with insulin resistance in Pro12 homozygotes (systolic, $p=0.001$; diastolic, $p<0.0001$), but not in Ala12 carriers (systolic, $p=0.75$; diastolic, $p=0.38$).

Results of Pro12Ala meal studies

Subjects

The characteristics of the 24 subjects (12 Pro12 homozygotes and 12 Ala12 carriers) are presented in Table 2. The six obese Pro12 homozygotes had a BMI of 31.6 kg/m² (31.3–32.4), whilst the six overweight Ala12 carriers had a BMI of 31.5 (30.8–32.2). The BMI of the six normal weight Pro12 homozygotes was 24.5 kg/m² (23.7–24.8), whilst that of the six normal weight Ala12 carriers was 24.6 kg/m² (24.2–24.9). There was no difference in body fat percentage, waist or hip measurements, fasting lipid measurements and blood pressure between the Pro12 homozygotes and Ala12 carriers. Unless otherwise stated, there was no difference between the findings of the lean and obese subgroups.

Glucose and insulin

As shown in Fig. 2, fasting plasma glucose and insulin concentrations were identical between the groups (fasting glucose concentrations: Pro12 homozygotes, 5.49 mmol/l [5.18–5.74] vs Ala12 carriers, 5.54 mmol/l [5.29–6.13], $p=0.28$; fasting insulin concentrations: Pro12 homozygotes, 92 pmol/l [76–130], Ala12 carriers, 100 pmol/l [64–115], $p=0.23$). The obese subjects in each group were more insulin-resistant than the lean subjects, based on fasting

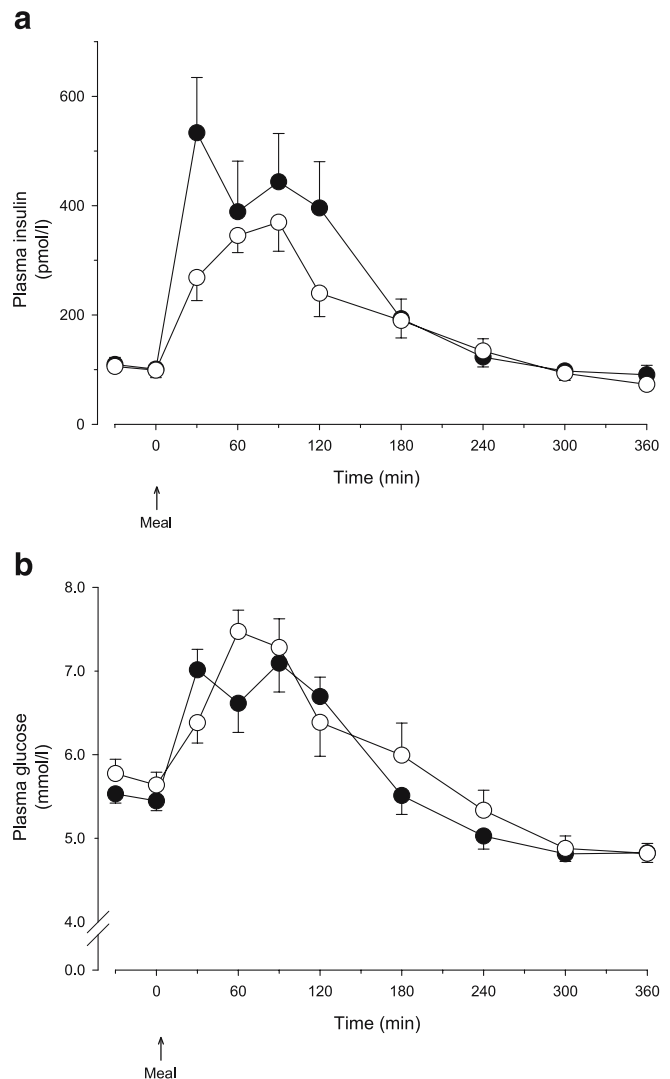


Fig. 2 Pro12Ala meal provocation study. Plasma insulin (**a**) and glucose (**b**) concentrations in response to a mixed meal (given at time 0) in Pro12 homozygotes (●) and Ala12 carriers (○). Glucose: $p=0.23$ (fasting), $p=0.004$ (postprandial) (**a**); insulin: $p=0.28$ (fasting), $p=0.35$ (postprandial) (**b**)

insulin and glucose concentrations and HOMA calculations (data not shown).

Postprandial glucose concentrations were the same in the Ala12 and Pro12 groups ($p=0.35$). In contrast, insulin concentrations were significantly lower in the postprandial period in Ala12 carriers than in Pro12 homozygotes ($p=0.004$).

Glucose uptake by forearm muscle and adipose tissue was the same in the Pro12 and Ala12 groups (Pro12 homozygotes, 0.51 µmol/min per 100 g tissue [0.34–0.81] vs Ala12 carriers, 0.67 µmol/min per 100 g tissue [0.45–0.73] [time-averaged AUC], $p=0.97$).

NEFA

Ala12 carriers had lower fasting and postprandial NEFA concentrations (–12.5%, $p=0.05$) (Fig. 3a). NEFA release

from subcutaneous abdominal adipose was not different between the groups (Fig. 3b). The Pro12 homozygotes showed a rather constant uptake of NEFA across the forearm, whereas the Ala12 group showed a significantly

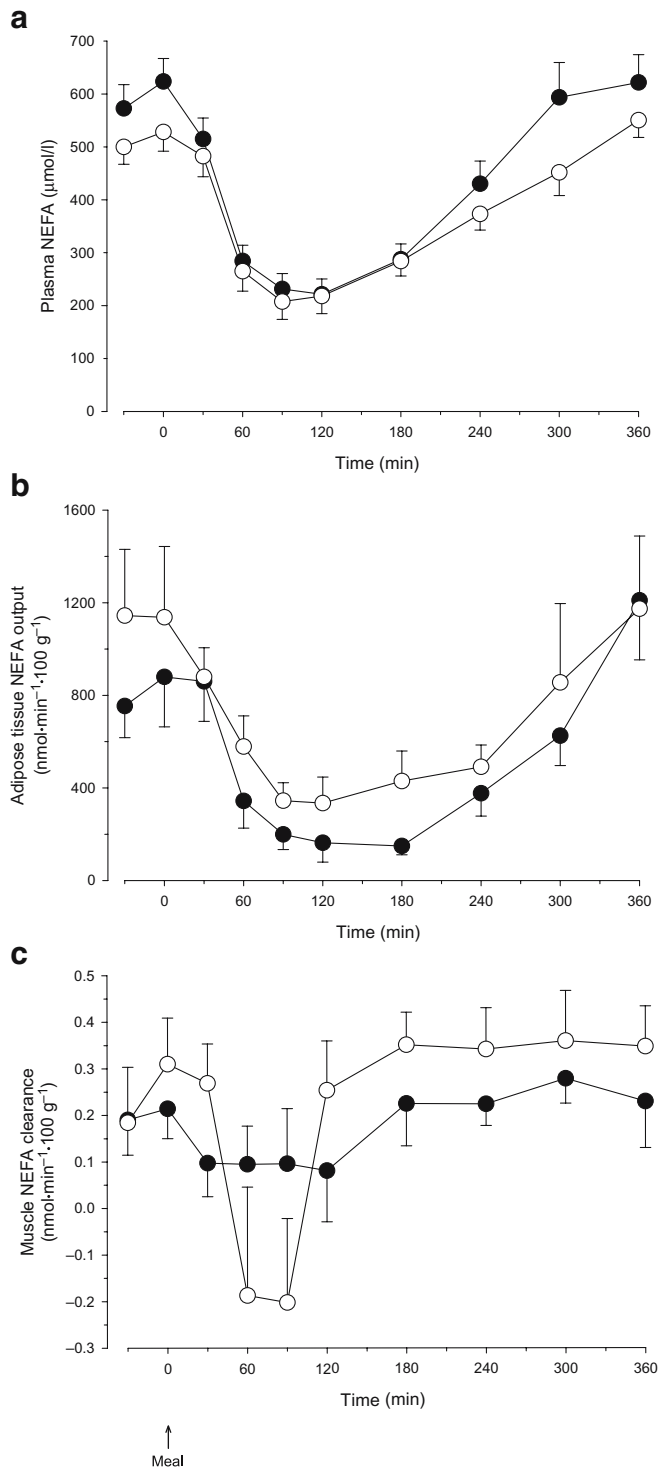


Fig. 3 Pro12Ala meal provocation study. NEFA metabolism in response to a mixed meal (given at time 0) in Pro12 homozygotes (●) and Ala12 carriers (○). **a** Plasma NEFA concentrations, $p=0.05$. **b** NEFA output from subcutaneous abdominal adipose tissue, $p=0.92$. **c** NEFA clearance from muscle, $p<0.01$

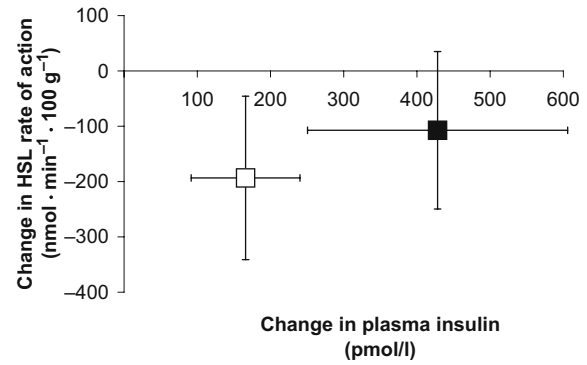


Fig. 4 Pro12Ala meal provocation study. Change in plasma insulin concentrations and change in HSL suppression 30 min after a mixed meal (given at time 0) in Pro12 homozygotes (■) and Ala12 carriers (○). 95% CIs are shown

decreased uptake of NEFA at the time of peak insulinaemia (Fig. 3c).

Hormone-sensitive lipase in adipose tissue

Figure 4 demonstrates the relationship between the increase in postprandial insulin concentrations (in the first 30 min after the meal), and the postprandial suppression of adipose tissue HSL rate of action (in the first 30 min after the meal), using bars showing the 95% CI. In the first 30 min, the insulin-induced suppression of HSL was significant after the meal in the Ala12 carriers, but the change was not significantly different from baseline (zero) in the Pro12 homozygotes. This is an important finding as it reflects differences in one of the most insulin-sensitive processes in adipose tissue, namely suppression of HSL activity, and implies that the Ala12 carriers had considerably greater insulin sensitivity in the early suppression of lipolysis. However, the overall HSL rate of action did not differ between the two groups ($p=0.81$).

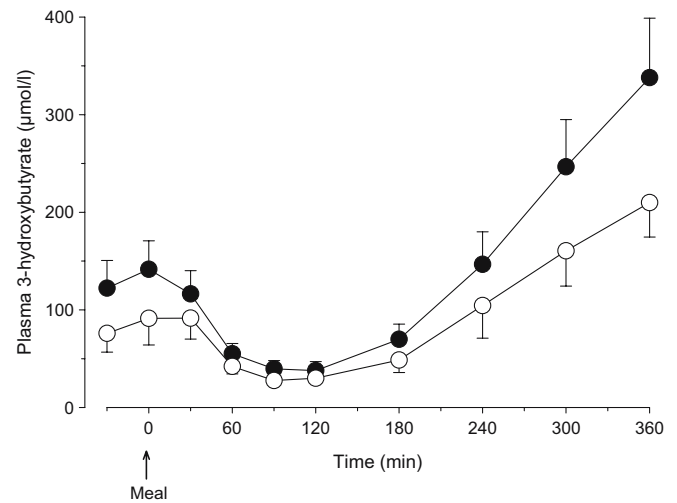


Fig. 5 Pro12Ala meal provocation study. Plasma 3-hydroxybutyrate concentrations in response to a mixed meal (given at time 0) in Pro12 homozygotes (●) and Ala12 carriers (○). $p=0.05$

3-Hydroxybutyrate

Plasma concentrations of 3-hydroxybutyrate, a reflection of hepatic fatty acid oxidation, followed a similar time course (Fig. 5) to plasma NEFA and were also lower in the Ala12 carriers than in Pro12 homozygotes (90 ± 18 vs 133 ± 19 $\mu\text{mol/l}$ [mean \pm SEM] [time-averaged AUC], $p=0.05$).

Triglycerides

Both fasting and postprandial plasma triglyceride concentrations were similar between the groups (data not shown). Triglyceride removal in adipose tissue ($p=0.86$) (data not shown) and forearm muscle (data not shown, $p=0.22$) did not differ between Pro12 homozygotes and Ala12 carriers.

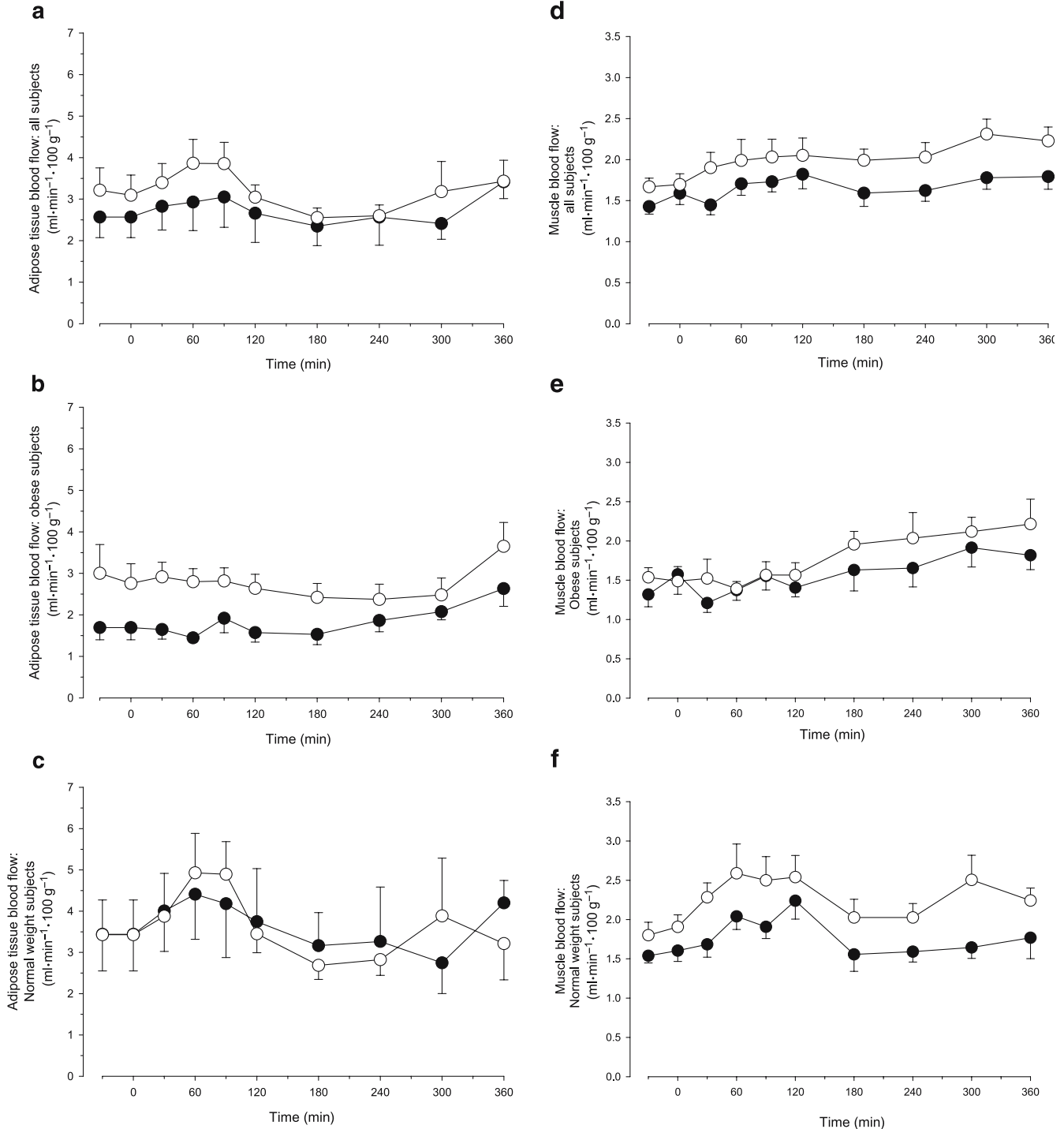


Fig. 6 Pro12Ala meal provocation study. Blood flow in response to a mixed meal (given at time 0) in Pro12 homozygotes (●) and Ala12 carriers (○). Adipose tissue blood flow is shown for all subjects, $p=0.06$ (a), for obese subjects, $p=0.03$ (b) and for normal-weight

subjects, $p=0.70$ (c). Forearm muscle blood flow is shown for all subjects, $p=0.03$ (d), for obese subjects, $p=0.30$ (e) and for normal-weight subjects, $p=0.04$ (f)

Stable isotopes

To quantify the proportion of fatty acids derived from the meal, we measured concentrations of [1-¹³C]palmitic acid in the triglyceride fraction and NEFA fraction. [¹³C] Palmitic acid concentrations increased postprandially in the triglyceride fraction (data not shown), and also in the NEFA fraction (data not shown), as would be expected after a meal containing labelled triglycerides. Notably, the rise in [1-¹³C]palmitic acid concentrations in both the NEFA and the triglyceride fraction were the same in both the Ala12 carriers and the Pro12 homozygotes.

The rate of appearance of exhaled ¹³CO₂ from [¹³C] palmitate did not differ between the Pro12 homozygotes and Ala12 carriers.

Adiponectin

Fasting plasma adiponectin concentrations were not different between Ala12 carriers and Pro12 homozygotes (mean±SEM, 6.5±1.0 vs 6.9±0.9 µg/ml, *p*=0.70).

Blood flow

Adipose tissue blood flow was higher in the Ala12 group compared with the Pro12 group (+17%), but this did not reach statistical significance (*p*=0.06) (Fig. 6a). This difference in adipose tissue blood flow was solely due to higher adipose tissue blood flow in overweight Ala12 subjects compared with overweight Pro12 subjects (+49%, *p*=0.03) (Fig. 6b); no difference could be seen between Ala12 and Pro12 subjects in the normal weight groups (+0%, *p*=0.70) (Fig. 6c).

Forearm muscle blood flow was also higher in the Ala12 than in the Pro12 group (+22%, *p*=0.03) (Fig. 6d). This difference in muscle blood flow was only seen in normal-weight Ala12 subjects compared with normal-weight Pro12 subjects (+29%, *p*=0.04) (Fig. 6e); no difference could be seen between overweight Ala12 and overweight Pro12 subjects (+14%, *p*=0.30) (Fig. 6f).

Discussion

This is one of the first studies to use a recruit-by-genotype approach to allow the prospective investigation of genotype–phenotype relationships in adipose tissue metabolism. Here, we show that, for investigation of the impact of a gene that is important in insulin resistance, such an approach was both feasible and successful.

Individuals recruited into the OBB should be considered as a highly motivated subset, although they are similar to those recruited into other large population-based studies (e.g. [24]). However, because the genotype frequencies of the polymorphism were in Hardy–Weinberg equilibrium, it

is likely that a representative sample of the population has been recruited here.

Although there were no overall metabolic differences between Pro12 homozygotes and Ala12 carriers (Table 2), the metabolic response to the presence of insulin resistance did differ, suggesting a gene–environment interaction. Using the OBB register, insulin sensitivity showed the expected correlations with plasma triglyceride, apoB and HDL-cholesterol concentrations among male Pro12 homozygotes, whereas the corresponding correlations were absent among male Ala12 carriers (Fig. 2). Thus, male Ala12 carriers seemed to be protected against the metabolic consequences of insulin resistance. Such findings prompted us to metabolically characterise the Pro12Ala polymorphism in greater detail.

The meal study of the Pro12Ala polymorphism recruited age-, sex- and BMI-matched subjects from the OBB register. Despite this close matching, we observed significant differences in a number of physiological variables. We found that Ala12 carriers had lower NEFA concentrations in the fasting and late postprandial periods than the Pro12 homozygotes. The use of a mixed meal as a physiological stimulus, rather than insulin clamp techniques, allows assessment of normal physiological variations in diurnal NEFA concentrations. Such elevation of plasma NEFA concentrations may play an important role in the pathogenesis of type 2 diabetes [25–27].

Postprandial excursions of plasma insulin concentrations were lower in Ala12 carriers, despite identical postprandial plasma glucose concentrations. This occurred despite identical fasting concentrations of insulin and glucose. This can only be interpreted as a sign of increased insulin sensitivity in the Ala12 carriers, which is consistent with the findings of other groups [5–9, 28].

The suppression of HSL activity in adipose tissue is an insulin-sensitive process, and occurs as insulin concentrations rise in the postprandial period. Thus, the measurement of HSL rate of action gives an estimate of the tissue-specific insulin sensitivity. Adipose tissue HSL in Ala12 carriers was suppressed rapidly in the postprandial period, whereas the postprandial suppression HSL of Pro12 homozygotes was not significantly different from baseline, despite higher insulin concentrations than in the Ala12 carriers (Fig. 4). By measuring systemic labelled glycerol concentrations during a clamp, Stumvoll and colleagues found that Ala12 carriers suppressed their systemic concentrations of glycerol at lower insulin concentrations than Pro12 homozygotes [28]. The mechanism of this is likely to be mediated by changes in HSL activity in adipose tissue, as shown by the tissue-specific measurements in our study.

One study found no difference in fasting NEFA concentrations between Pro12 homozygotes and Ala12 carriers [29]. However, by careful phenotyping of matched subjects, we did find subtle differences in NEFA metabolism between Pro12 homozygotes and Ala12 carriers. Fasting and postprandial NEFA concentrations were lower in Ala12 carriers, despite unchanged NEFA release from

subcutaneous abdominal adipose tissue. Could a difference in the generation of NEFAs from dietary triglycerides account for the difference in plasma NEFA concentrations observed? By measuring plasma concentrations of stable isotopes derived from the meal containing [1,1,1-¹³C] tripalmitin, we were able to assess the generation of NEFAs from dietary triglycerides; there was no difference between Ala12 carriers and Pro12 homozygotes. This use of stable isotopes addressed the question of whether there was an increased spillover of fatty acids from the intravascular lipolysis of chylomicron triglycerides. These data did not support that possibility.

Tissue blood flows were also affected by the Pro12Ala polymorphism. Adipose tissue blood flow tended to be higher in Ala12 carriers compared with the Pro12 homozygotes. This difference was due to higher adipose tissue blood flow in the obese Ala12 subgroup. We have previously shown a strong relationship between adipose tissue blood flow and insulin sensitivity [30], and these findings support the notion that the adipose tissue of Ala12 carriers is more insulin-sensitive than that of Pro12 homozygotes. There was a small but statistically significant increase in forearm muscle blood flow in the Ala12 carriers, but this was accounted for by differences between the lean subgroups. We have previously shown that increasing insulin sensitivity by PPAR γ activation increases adipose tissue blood flow, but does not affect muscle blood flow [12]. Thus, the explanation for the observed increase in muscle blood flow among lean Ala12 carriers is unclear. PPAR γ activation is known to modify vascular responsiveness *in vivo* [31–33] and *in vitro* [34–36], but the effect of PPAR γ 2 Pro12Ala polymorphism has never been assessed previously. Thus, Ala12 carriers appear to have a constitutively higher blood flow compared with Pro12 homozygotes. Such genotype-specific difference in nutritive blood flow is likely to impact on the metabolic function of adipose tissues and muscle [37, 38].

Some authors have suggested that the Pro12Ala polymorphism influences adipose tissue distribution, finding that Pro12 homozygotes have centrally distributed fat, whilst Ala12 carriers have increased peripheral fat [39, 40]. In the OBB database, skinfold and waist:hip measurements were not significantly different between the Pro12 homozygotes and Ala12 allele carriers, irrespective of sex.

Removal of NEFAs from the circulation occurs in muscle and liver. Here, we show that Ala12 carriers modulate their muscle NEFA clearance from fasted to fed and back to the fasted state (Fig. 3c). However, the muscle NEFA uptake of Pro12 homozygotes shows little change at the time of peak postprandial insulinemia. This flexibility of NEFA metabolism across the forearm muscle suggests a genotype-specific difference in skeletal muscle NEFA handling. At first glance, such an effect is difficult to reconcile, considering that the Pro12Ala polymorphism is specific to PPAR γ 2, whilst it is PPAR γ 1 which is found in skeletal muscle [41]. It is therefore likely that the effect in skeletal muscle is secondary to changes in systemic NEFA con-

centrations or other metabolic mediators. Such metabolic flexibility reflects the ability of tissues to switch from the use of one fuel to another and has been suggested as a more important marker of insulin sensitivity than absolute concentrations of metabolites [42, 43].

3-Hydroxybutyrate is generated by the liver from fatty acid oxidation and indirectly reflects NEFA handling in the liver; this was lower in Ala12 carriers than in Pro12 homozygotes. Whether this is the primary reason for the difference in plasma NEFA concentrations, or whether the lower NEFA oxidation in the liver merely reflected lower plasma NEFA concentrations is unclear. Interestingly, the measure of whole-body fatty acid oxidation, the rate of appearance in expired air of ¹³CO₂ from [¹³C]palmitate, did not differ between Ala12 carriers and Pro12 homozygotes. Thus, the lower rate of fatty acid oxidation in the liver of Ala12 carriers may have been compensated for by a lower rate of fatty acid oxidation in the skeletal muscle.

Adiponectin is known to increase fatty acid oxidation and thus might be expected to influence NEFA concentrations. However, reports of the differences in plasma adiponectin concentrations between Ala12 carriers and Pro12 homozygotes are conflicting [44–46]. The differences are perhaps explained by the race of the subjects studied: among Japanese subjects, Ala12 carriers have lower plasma adiponectin concentrations than Pro12 homozygotes [44, 45], whilst among Caucasian subjects there is no difference between Pro12 homozygotes and Ala12 carriers [46]. Our study of a Caucasian population is consistent with these findings.

The use of a mixed meal as the physiological stimulus to assess the effects of metabolic provocation has benefits compared with non-physiological insulin doses, as occurs when hyperinsulinaemic clamps are used. Many studies look at the association between measures of fasting insulin sensitivity (e.g. HOMA-IR) and the Pro12Ala polymorphism. We can see that in response to the physiological stimulus of a mixed meal, the main effects on insulin sensitisation are observed in the postprandial period. Another advantage of using a physiological stimulus of a meal is that the transition from the fed to the fasted state allows us to explore features of metabolic flexibility, an important characteristic lost in insulin resistance [42, 43]. There were subtle differences in metabolism between healthy Pro12 homozygotes and Ala12 carriers, including differences in plasma NEFA concentrations and tissue blood flow, along with increased insulin sensitivity. These tissue-specific physiological findings are consistent with the epidemiological observation that the PPAR γ Ala12 allele protects against the development of type 2 diabetes.

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