A partial loss-of-function variant in *AKT2* is associated with reduced insulin-mediated glucose uptake in multiple insulin sensitive tissues: a genotype-based callback positron emission tomography study

Running title: *AKT2* variant and tissue glucose uptake

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Rare fully penetrant mutations in *AKT2* are an established cause of monogenic disorders of glucose metabolism. Recently, a novel partial loss-of-function *AKT2* coding variant (p.Pro50Thr) was identified that is nearly specific to Finns (frequency 1.1%), with the low-frequency allele associated with an increase in fasting plasma insulin level and risk of type 2 diabetes. The effects of p.Pro50Thr on insulin-stimulated glucose uptake (GU) in the whole body and in different tissues have not previously been investigated. We identified carriers (N=20) and matched non-carriers (N=25) for this allele in the population-based METSIM study and invited these individuals back for positron emission tomography study with [^{18}F]-fluorodeoxyglucose during euglycemic hyperinsulinemia. When we compared p.P50T/*AKT2* carriers to non-carriers, we found a 39.4% reduction in whole body GU (P=0.006) and a 55.6% increase in the rate of endogenous glucose production (P=0.038). We found significant reductions in GU in multiple tissues: skeletal muscle (36.4%), liver (16.1%), brown adipose (29.7%), and bone marrow (32.9%), and increases of 16.8-19.1% in 7 tested brain regions. These data demonstrate that the P50T substitution of *AKT2* influences insulin-mediated GU in multiple insulin sensitive tissues, and may explain, at least in part, the increased risk of type 2 diabetes in p.P50T/*AKT2* carriers.

KEY WORDS: AKT/pkb, glucose uptake, insulin resistance, genetics, positron emission tomography

Many large-scale exome and genome sequencing studies currently are underway to identify low-frequency and rare genetic variants associated with human diseases and traits. Large samples typically are required to obtain convincing association evidence for such variants. Once a rare-variant association is identified, investigators may call back carriers and non-carriers of the associated variant from the study population and undertake additional phenotyping to help understand disease mechanism. Such phenotyping might not have been considered at study outset or might have been too costly to undertake in the full study sample. Finland provides an ideal base for genotype callback studies. The history of Finland, with recent population bottlenecks, has resulted in increased frequency of genetic variants that are rare elsewhere, including non-synonymous and particularly loss-of-function variants (1). Further, Finland boasts a well-educated population strongly supportive of biomedical research. In our present study we applied this callback approach to investigate the effects of a partial loss-of-function variant p.Pro50Thr (rs184042322) *AKT2* (*V-AKT Murine Thymoma Viral Oncogene Homolog 2*) (p.P50T/*AKT2*) on the rates of glucose uptake (GU) in whole body and in multiple insulin sensitive tissues to understand the mechanisms explaining increased risk of type 2 diabetes in p.P50T/*AKT2* carriers.

The AKT2 protein plays a key role in the conserved phosphoinositide 3-kinase (PI3K) signalling pathway, downstream of the insulin receptor, and mediates the physiological effects of insulin in several tissues including liver, skeletal muscle, and adipose tissue (2-4). Additionally, *AKT2* is expressed in the bone marrow, heart, brain, small intestine, and kidney. Mice deficient in Akt2 develop hyperglycemia, hyperinsulinemia, insulin resistance, age-dependent loss of adipose tissue, and diabetes in males (1,5).

In humans, rare penetrant mutations in the *AKT2* gene encoding AKT Serine/Threonine Kinase 2 have been previously associated with monogenic disorders of glucose metabolism. The first p.Arg274His mutation described in a single family showed autosomal dominant inheritance of severe insulin resistance and diabetes and disrupted insulin signalling in cultured cells. Individuals with this

loss-of-function mutation were unable to phosphorylate glycogen synthase kinase 3 (GSK3) in an *in vitro* kinase assay (6). In contrast, another mutation, p.Glu17Lys, caused severe fasting hypoinsulinemic hypoglycemia. AKT2 p.Glu17Lys was constitutively located at the plasma membrane (7) and overexpression induced translocation of GLUT4 to the plasma membrane (8).

In a recent meta-analysis of exome genotype data on 33,231 non-diabetic individuals of European ancestry, investigators demonstrated that carriers of the low-frequency amino acid substitution p.P50T/*AKT2* had on average a 12% (95% confidence interval 7-18%, P=1.0 x 10^{-9}) increase in fasting insulin level, and an increased risk of type 2 diabetes (allele-specific odds ratio 1.05, P=8.1 x 10^{-5}) (9). *In vitro* studies demonstrated the variant protein leads to a partial loss of AKT2 phosphorylation at its activation sites (Thr308 and Ser473), suggesting impaired AKT2 signalling and a reduced ability to phosphorylate its downstream target glycogen synthase kinase 3 beta (GSK3β) (9). The p.P50T/*AKT2* variant was found at a frequency of 1.1% in Finns, but was present at much lower frequencies in other ancestries (MAF 0.2% in non-Finnish Europeans and ≤0.01% in African American, Asian, and Hispanic individuals) making Finland the ideal place for more detailed genotype-phenotype investigations.

RESEARCH DESIGN AND METHODS

The METSIM positron emission tomography (PET) studies

Study participants

We selected male participants from the ongoing METSIM follow-up study with (N=20, 1 homozygous, 19 heterozygous) and without (N=25) p.P50T/*AKT2* and matched for age and BMI (10,11). They fulfilled the following inclusion criteria: age from 50 to 75 years, BMI from 20 to 40 kg/m^2, and a non-diabetic oral glucose tolerance test. We applied the following exclusion criteria: diabetes, a chronic disease that could affect glucose metabolism (e.g. liver, kidney, thyroid, cancer),

abusive use of alcohol, and any chronic medication that could affect glucose metabolism (e.g. steroids, beta-blockers, thiazide diuretics, antipsychotics, antidepressants). We performed PET studies at the PET Centre of the University of Turku, Finland. Assuming the sample sizes of 20 and 25 in the two groups, we had 80% power at significance level α=0.05 to detect a 30% difference in the means of skeletal muscle GU based on previous studies performed at the Centre. The Ethics Committee of the Hospital District of Southwest Finland approved the study protocol. The study was conducted according to the principles of the Declaration of Helsinki. All participants gave written informed consent prior to participation in the study.

Genotyping

We originally genotyped the participants of the METSIM study on the Illumina HumanExome Beadchip (9). We confirmed the p.P50T/*AKT2* genotypes with TaqMan Allelic Discrimination Assays (Applied Biosystems) for PET study participants.

Hyperinsulinemic euglycemic clamp

We performed an hyperinsulinemic euglycemic clamp after an overnight fast of 10-12 hours. Two catheters were inserted in veins of opposite forearms; one in the right antecubital vein for blood sampling and another in the left forearm for glucose and insulin infusions and radiotracer injection. To obtain arterialized venous plasma, the right arm was warmed. After catheterization, we collected baseline samples and performed the hyperinsulinemic euglycemic clamp as previously described (12) with the insulin infusion rate of 40 mU m^{-2} body surface area min^{-1} (Actrapid, Novo Nordisk, Copenhagen, Denmark). We maintained euglycemia by moderating the rate of 20% glucose infusion based on the plasma glucose level measured every 5-10 minutes. We reported the rates of whole body GU (M-value) as the average of 20-minute intervals between 60-140 minutes after the start of insulin infusion.

Glucose uptake measurements using PET/CT during the hyperinsulinemic euglycemic clamp

We quantified the rates of tissue specific GU using the PET/CT (Discovery 690, General Electric (GE) Medical systems, Milwaukee, WI, USA), with 2-deoxy-2-[^{18}F] fluoro-D-glucose (^{18}F-FDG) as tracer. The method of producing the tracer has been previously described (13). After reaching a steady euglycemia (69 ± 15 min from the start of insulin infusion), we injected participants with 152 ± 10 MBq of ^{18}F-FDG and started PET scanning. The scanned regions were heart (40 min), liver (15 min), upper abdomen (15 min), thigh skeletal muscle (15 min), neck (10 min), and brain (10 min). We performed all PET measurements blinded to *AKT2* genotype.

Endogenous glucose production (EGP)

We collected a urine sample immediately after GU measurements, and measured the amount of radiotracer lost into urine using an isotope dose calibrator (Model VDC-205, Comecer Netherlands, Joure, Netherlands). We assessed EGP by subtracting glucose infusion rate from rate of glucose disposal derived from ^{18}F-FDG consumption (14). The liver produces ~80% of EGP and the kidney ~20% (15).

Non-brain PET glucose uptake

Before analysis, we corrected imaging data for dead time, decay, and photon attenuation. To determine the input function, we calculated a blood-time activity curve by combining arterial blood activity data from the PET images (first 10 min after injection) with measurements made from arterialized venous blood plasma samples collected at nine time points (5, 10, 20, 30, 40, 47.5, 62.5, 75, and 85 min after injection) during the scanning (16). We determined plasma activity using an automatic gamma counter (Wizard 1480 3, Wallac, Turku, Finland). We derived tissue activity and fractional uptake (Ki) of the tracer from graphical analyses (17) applying the Carimas Software (Version 2.9, Turku PET Centre, downloadable at http://www.turkupetcentre.fi). We used a segmenting tool for myocardium to include the left ventricle wall and septum in the analysis; for other

tissues the regions of interest (ROIs) were drawn manually. For skeletal muscle analysis ROIs were drawn to include the medial parts of quadriceps femoris muscle of both thighs; for the liver a section of the right lobe free of large vessels was chosen. The same researcher (A.L.-R.) performed analyses blinded, and estimated the rates of skeletal muscle and liver GU twice for the first 24 participants. The Pearson correlation between the two measurements was 0.99 for skeletal muscle and 0.92 for liver.

We report the average of several ROIs for different adipose tissue types, with subcutaneous adipose tissue ROIs positioned around waistline, visceral adipose tissue ROIs in intraperitoneal cavity, and brown adipose tissue ROIs in supraclavicular areas on both sides of the neck. Bone marrow ROIs were drawn inside the body of both femoral bones and reported as their average.

Brain PET glucose uptake

We carried out preprocessing and statistical analyses of the brain-PET-images with the SPM 12 software (http://www.fil.ion.ucl.ak.uk/spm/). We first normalized PET images into an in-house ^{18}F-FDG template according to the Montreal Neurological Institute standard using linear and nonlinear transformations, and smoothed with a Gaussian kernel with 8-mm full-width at half-maximum. Next, we quantified the voxelwise fractional uptake rate (FUR) as the ratio of tissue time activity and integral of plasma activity from time 0 to the time of the scan. We compared voxelwise between-groups differences in FUR using a nonparametric full volume analysis in the SnPM13 toolbox (http://warwick.ac.uk/snpm). We constructed anatomical ROIs in the brain lobes, midbrain, limbic system, and cerebellum in a manner parallel to that for the other tissues.

Calculation of tissue-specific glucose uptake

To assess the rates of tissue-specific GU (μmol kg^{-1} min^{-1}), we multipled tissue fractional uptake by plasma glucose concentration during scanning and divided by tissue density, and a previously established lumped constant: 1.2 for skeletal muscle, 1.0 for myocardium and liver, 1.14 for adipose

tissue, 1.1 for intestine, and 0.65 for brain (18-24). The lumped constant for bone marrow has not been defined, so we adopted the previously used value of 1.0 (25) to compare the results between groups.

Laboratory measurements

We measured plasma glucose in duplicates using the glucose oxidase method (Analox GM9 Analox Instruments, London, UK) in the fasting state and during the clamp. We determined plasma insulin levels in the fasting state and at 30 min intervals after the start of insulin infusion until the end of clamp using an automated electrochemiluminescence immunoassay, ECLIA (Cobas 8000, Roche Diagnostics, Mannheim, Germany). We measured serum free fatty acid (FFA) levels in the fasting state and at 60 min intervals during the clamp with an enzymatic colorimetric method assay (NEFA-HR2, ACS-ACOD, Wako Chemicals, Neuss, Germany; Cobas 8000 c502 Analyzer, Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analyses

We carried out data analyses with IBM SPSS 21.0 for Windows (Chicago, IL, USA). We give the results for continuous variables as means ± SD. We logarithm transformed variables with skewed distribution (insulin, triacylglycerol, FFA, GU in subcutaneous and visceral adipose tissue) prior to statistical analyses. We assessed he differences between the groups by the independent samples t-test for continuous variables and χ^2 test for discrete variables. We used linear regression to adjust the results for outside temperature in the previous 30, 14, and 7 days in statistical analyses of brown adipose tissue GU. We assessed the correlation between different measures of GU by the Spearman correlation coefficient. We used the Fisher´s r-to-z transformation to compare correlation coefficients in carriers and non-carriers of the p.P50T/*AKT2*. The threshold for statistical significance was set at α=0.05.

RESULTS

The euglycemic hyperinsulinemic clamp and PET study

Characteristics of the participants. Characteristics of the p.P50T/*AKT2* carriers (N=20, 1 homozygous, 19 heterozygous) and non-carriers (N=25) without chronic diseases are presented in Table 1. These two groups of participants were matched for age and BMI and did not differ significantly by age, BMI, or fasting glucose. As expected fasting insulin was higher in the p.P50T/*AKT2* carriers than in the non-carriers. We pooled the single p.P50T/*AKT2* homozygous carrier with heterozygous carriers in all statistical analysis because the homozygous carrier was not an outlier among the group of carriers.

Whole body glucose uptake (GU), glucose disposal (Rd), and endogenous glucose production (EGP) (Figure 1A). Whole body GU was assessed by the euglycemic hyperinsulinemic clamp-based M-value and the Rd by the ^{18}F-FDG disappearance rate (20). To verify the quality of the euglycemic hyperinsulinemic clamp, we compared the mean glucose levels during the clamp in p.P50T/*AKT2* carriers and non-carriers; we observed essentially no difference between the two groups (5.0 ± 0.4 and 5.0 ± 0.2 mmol/l, *P*=0.53). The rates of whole body GU (17.6 ± 10.3 vs 29.2 ± 15.2 µmol/kg/min, *P*=0.006) and glucose disposal (Rd) (25.6 ± 9.9 vs 33.1 ± 11.9 µmol/kg/min, *P*=0.029) were lower in p.P50T/*AKT2* carriers compared to non-carriers (Figure 1A). EGP during the clamp was higher in p.P50T/*AKT2* carriers than in non-carriers (9.0 ± 2.6 vs 5.8 ± 6.9 µmol/kg/min, *P*=0.038).

Tissue specific glucose uptake (Figure 1B and 1C)**.** We assessed GU in different tissues using the euglycemic hyperinsulinemic clamp and PET. We observed lower rates of GU in carriers of the p.P50T/*AKT2* variant compared to non-carriers in skeletal muscle (23.9 ± 14.1 vs 37.5 ± 20.7 µmol/kg/min, *P*=0.012), liver (21.0 ± 5.1 vs 25.1 ± 6.6 µmol/kg/min, *P*=0.030), brown adipose tissue (11.7 ± 5.1 vs 16.7 ± 6.9 µmol/kg/min, P=0.004), and bone marrow GU (13.3 ± 5.4 vs 19.8 ± 8.8 µmol /kg/min, *P*=0.004) (Figure 2), but did not observe significant differences in subcutaneous

adipose tissue (11.3 ± 4.1 vs 12.7 ± 5.8 µmol/kg/min, *P*=0.488), visceral adipose tissue (17.3 ± 6.4 vs 20.9 ± 8.5 µmol/kg/min, *P*=0.157), myocardium (34.2 ± 16.8 vs 35.0 ± 12.6 µmol/100 g/min, *P*=0.870), duodenum (31.9 ± 7.0 vs 31.7 ± 7.1 µmol/kg/min, *P*=0.931), or jejunum (33.2 ± 7.0 vs 32.4 ± 7.2 µmol/kg/min, *P*=0.711). We observed higher rates of GU in the p.P50T/*AKT2* carriers than in non-carriers in all seven analyzed brain regions (*P*=0.001) (Figure 2).

FFA levels in fasting and during the clamp. Fasting FFA levels did not differ between carriers and non-carriers of p.P50T/*AKT2* (0.43 ± 0.16 vs 0.39 ± 0.16, *P*=0.360). However, FFA levels were higher during hyperinsulinemia at 60 min in carriers than in non-carriers of p.P50T/*AKT2* (0.16 ± 0.12 vs 0.09 ± 0.05 mmol/l, *P*=0.024).

Correlations between the rates of whole body (Figure 3A) and brain (Figure 3B) GU with tissue specific GU and EGP in carriers and non-carriers of* p.P50T/*AKT2. The differences in the rates of GU across several tissues between carriers and non-carriers of p.P50T/*AKT2* we observed prompted us to investigate the correlations of the rates of GU separately in carriers and non-carriers of p.P50T/*AKT2*. Whole body GU correlated positively with skeletal muscle GU (r=0.92 vs r=0.90), bone marrow GU (r=0.74 vs r=0.85), subcutaneous fat GU (r=0.59 vs 0.40), liver GU (r=0.41 vs r=0.46), and negatively with brain GU (r=-0.56 vs r=-0.66) in both non-carriers and carriers of p.P50T/*AKT2*, respectively (Figure 3A). Correlations of the rates of whole body GU with brown fat GU (r=0.80 vs r=0.36, *P*=0.023) and endogenous glucose production in the liver (r=-0.41 vs -0.08, *P*=0.276) were substantially weaker among the carriers than among non-carriers of p.P50T/*AKT2*. Whole body GU correlated weakly with heart muscle GU and jejunum GU without any substantial difference between the non-carriers and carriers of p.P50T/*AKT2*. Correlations of brain GU with EGP (r=0.68 vs 0.05, *P*=0.016), and bone marrow GU (r=-0.24 vs r=-0.84, *P*=0.002) were significantly different between the non-carriers and carriers of p.P50T/*AKT2* (Figure 3).

DISCUSSION

Our genotype-based callback PET study demonstrates that a low-frequency partial loss-of-function p.P50T/*AKT2* variant, nearly unique to Finns, and probably originating from a recent bottleneck in the 16th century in the settlement of Eastern Finland (1), is associated with significantly decreased GU in whole body and in multiple insulin sensitive tissues. This is consistent with our previous study (9) demonstrating that insulin levels were increased in carriers of p.P50T/*AKT2* as a compensatory mechanism for insulin resistance. The increase in insulin levels was substantially less in carriers of p.P50T/*AKT2* compared to carriers of the p.Arg274His/*AKT2* loss-of-function mutation previously reported (6).

Activation of AKT2 is associated with translocation of glucose transporter type 4 (GLUT4) from intracellular storage vesicles to the cell surface (26,27). AKT2 is the major isoform of AKT and is abundantly expressed in skeletal muscle (8,9). Insulin-stimulated AKT2 activation leads to inactivation of glycogen synthase kinase 3 β (GSK3β) (3,28), resulting in increased glycogen synthesis. Moreover, gene silencing experiments have provided evidence that AKT2 is indispensable for insulin action on glucose uptake and glycogen synthesis in human skeletal muscle cells (29). The present study shows that *in vivo* skeletal muscle GU was reduced by 36% (P=0.012) in the p.P50T/*AKT2* carriers compared to non-carriers. This could be explained, at least in part, by reduced activity of the low-frequency p.P50T/*AKT2* variant, in agreement with our previous finding of impaired insulin signaling in HeLa cells and human liver HuH7 cells for the variant (9). Collectively, these findings demonstrate that AKT2 is an important determinant of insulin sensitivity in human skeletal muscle.

The liver plays an important role in maintaining normal glucose levels by regulating EGP (gluconeogenesis) and glycogenolysis (glycogen breakdown). Additionally, the kidney produces about 20% of EGP (15). Normally, insulin suppresses EGP and inhibits the genes encoding gluconeogenesis and redirects newly synthesized glucose-6-phosphate to glycogen (3). We found that

EGP was significantly increased and liver GU decreased in the carriers of the *AKT2* variant compared to non-carriers indicating liver insulin resistance. AKT2 plays an important role in the regulation of liver and kidney (29) insulin sensitivity. AKT2 phosphorylates and inhibits Foxo1, a key regulator of EGP (30). Our findings agree with the results observed in mice deficient in Akt2, which demonstrated a significant failure of insulin to suppress EGP (2). Additionally, we found that liver GU was decreased in the carriers of the p.P50T/*AKT2* variant compared to non-carriers. This could be due to impaired insulin signaling attributable to the p.P50T/*AKT2* variant which results in subnormal inactivation of GSK3β. Other mechanisms, independent of GSK3β suppression, could also play a role, as recently suggested (3).

Activation of AKT2 enhances GLUT4 translocation and the rates of GU similarly in adipose tissue and skeletal muscle (6). GU into the white adipose tissue is relatively minor, accounting for only 5–10% of whole body GU during insulin-stimulated states, suggesting that white adipose tissue does not have a major quantitative role in postprandial glucose metabolism (31,32). We did not find a statistically significant difference between the carriers and non-carriers of p.P50T/*AKT2* in the rates of GU in subcutaneous or visceral adipose tissue, although the rates of GU were slightly lower in variant carriers than in non-carriers. The carriers and non-carriers of p.P50T/*AKT2* had similar weight, BMI, waist circumference, and fat percentage, making it unlikely that obesity, central obesity, or fat mass could have an effect on the rates of adipose tissue GU. However, we found that the levels of FFAs were higher during the clamp at 60 min in carriers of p.P50T/*AKT2* than in non-carriers, suggesting that insulin's inhibitory effect on adipose tissue lipolysis was impaired in carriers of p.P50T/*AKT2* (33).

Brown adipose tissue is mainly located in the supraclavicular region in adult humans, has high mitochondrial content and insulin sensitivity, rich vasculature, and is activated by cold exposure (34). We observed that the rates of brown adipose tissue GU were significantly lower in p.P50T/*AKT2* variant carriers than in non-carriers, not surprising since hyperinsulinemia increases GU in brown

adipose tissue up to 5-fold compared to the fasting state (22). A recent study demonstrated that mice lacking adipocyte *Akt1* and *Akt2* had no discernible subcutaneous or brown adipose tissue, and developed lipodystrophy, severe insulin resistance, and hepatomegaly (35). However, p.P50T/*AKT2* variant carriers in our study did not have lipodystrophy, reduced fat mass, or elevated liver enzymes (Table 1). This is consistent with our previous *in vitro* studies showing that p.P50T/*AKT2* is only a partial loss-of-function variant (9). To assess the effects of outside temperature on brown adipose tissue activity, we adjusted statistical analyses for the mean temperatures in the previous 30, 14, and 7 days; these adjustments had no meaningful effect on our results.

Bone marrow of the femoral diaphysis in adults consists mostly of adipocytes. Femoral bone marrow 'yellow' adipose tissue, consisting of a moderate number of mitochondria, has intermediate metabolic activity compared to brown and white adipose tissue. It is still unclear whether 'yellow' adipose tissue constitutes a homogeneous population of brown or white adipocytes or is a heterogeneous population of both types of adipose tissue cells (36). We have recently shown that femoral bone marrow insulin stimulated GU correlated with whole body insulin sensitivity in elderly women (37). Here we observed a significant correlation of the rates of femoral bone marrow insulin-stimulated GU with the rates of skeletal muscle GU in men. Therefore, it is possible that femoral bone marrow exhibits a similar impairment in GU as skeletal muscle attributable to impaired AKT2 signaling.

Glucose is the major source of energy in the brain. Reduced brain insulin uptake has been postulated to lead to a decrease in brain insulin sensitivity to stimulate central nervous system pathways (38). In a previous PET study, brain GU was similar in participants with impaired glucose tolerance and healthy individuals in the fasting state but increased by 18% during hyperinsulinemia in participants with impaired glucose tolerance and not in healthy participants, suggesting that in insulin resistant states brain GU is paradoxically increased (39). Similarly, in another PET study, brain GU during hyperinsulinemia was increased in obese but not in non-obese participants (40). In

our study, brain GU was greater in p.P50T/*AKT2* variant carriers compared to non-carriers by 16.8 – 19.1% in different regions of the brain. These results suggest that both acquired (impaired glucose tolerance, obesity) and inherited (p.P50T/*AKT2*) insulin resistance may lead to the increased rates of brain GU. The molecular mechanism of this phenomenon is poorly understood. A recent study in rats demonstrated that ^{18}F-FDG PET signal reflects GU not only in neurons, but also in astrocytes (41). Moreover, the insulin signaling cascade is functional in primary human astrocytes, and increases Akt serine 473 phosphorylation (42). We plan to investigate the role of p.P50T/*AKT2* in astrocyte GU in *in vitro* studies.

Interestingly, correlation of brain GU with EGP was significantly different between the non-carriers and carriers of p.P50T/AKT2 (r=0.68 vs 0.05, *P*=0.016). A previous study in rats demonstrated that hypothalamic insulin signaling has significant effects on liver glucose production during hyperinsulinemia (43). Our results suggest that in p.P50T/*AKT2* carriers, insulin regulation of EGP is lost, resulting in increased glucose production by the liver and kidney during hyperinsulinemia.

The main source of energy in the heart is FFAs, but energy can also be derived from other sources including glucose, pyruvate, and lactate. Therefore, it is not surprising that we did not observe significant differences in myocardial GU between carriers and non-carriers of the p.P50T/*AKT2* variant, in contrast to the substantial differences observed in skeletal muscle. A recent study demonstrated that insulin was able to increase GU by almost 3-fold in duodenum and jejunum in normal weight non-obese participants, but obese non-diabetic participants showed no response to insulin, implying insulin insensitivity in the small intestine (23). Although *AKT2* is expressed in small intestine we did not observe any difference in GU into duodenal or jejunal mucosa between carriers and non-carriers of p.P50T/*AKT2*.

The strengths of our study are a careful matching of the study groups for sex (all male), age, and BMI, strict inclusion criteria to exclude participants with diseases and drug treatments which could

have an effect on tissue-specific GU, and that all study procedures in Turku were performed blinded to the genotype of the participants. The tissue specific differences in the kinetics of ^{18}F-FDG and glucose in skeletal muscle, adipose tissue, liver and intestine were corrected using lumped constant validated in our laboratory in healthy participants during similar clamp conditions. The primary limitation of the study is that it included only middle-aged and elderly men; it would be interesting to repeat our study in women and younger individuals.

In conclusion, our genotype-based callback study demonstrates a significant decrease of the insulin-mediated glucose uptake in skeletal muscle, liver, brown adipose tissue, and bone marrow, and an increase of GU in the brain in the carriers of the p.P50T/*AKT2* variant compared to the non-carriers of this variant. These changes in glucose uptake may explain, at least in part, the increased risk of type 2 diabetes in p.P50T/*AKT2* carriers. Our study also demonstrates the value of genotype-based callback studies and the practicality of PET as an informative, non-invasive method to characterize the function of genetic variants of interest .

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such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of Interest

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Table 1 - Clinical and laboratory characteristics of the p.P50T/*AKT2* non-carriers and carriers who participated in the METSIM positron emission tomography studies

Variable	Non-carriers (N=25) Mean ± SD	Carriers (N=20) Mean ± SD	*P* value
Age, years	63.9 ± 4.8	61.9 ± 6.3	0.23
Height, cm	176.9 ± 5.3	174.2 ± 5.5	0.10
Weight, kg	87.4 ± 10.2	86.1± 11.6.2	0.70
Body mass index, kg/m^2	28.1 ± 3.4	28.7 ± 3.4	0.60
Waist, cm	100.7 ± 8.9	100.3 ± 8.7	0.88
Fat mass, %	29.0 ± 7.0	28.0 ± 7.0	0.60
Systolic blood pressure, mmHg	133.8 ± 14.1	137.3 ± 15.9	0.44
Diastolic blood pressure, mmHg	86.4 ± 10.1	86.6 ± 8.5	0.94
Fasting plasma glucose, mmol/L	6.0 ± 6.5	6.1 ± 0.3	0.28
Fasting insulin, mU/l	9.4 ± 5.6	17.8 ± 10.2	0.003
LDL cholesterol, mmol/l	3.30 ± 0.96	2.92 ± 1.09	0.21
HDL cholesterol, mmol/l	1.51 ± 0.38	1.33 ± 0.37	0.12
Total triglycerides, mmol/l	1.12 ± 0.50	1.48 ± 1.04	0.26
Alanine transferase, U/L	29.7 ± 13.6	32.6 ± 17.8	0.58
Creatinine, µmol/l	85.3 ± 10.5	85.5 ± 12.6	0.96

Total triglycerides and ALT were log-transformed to calculate *P* value. LDL, low density lipoprotein; HDL, high density lipoprotein.

Figure captions

Figure 1. Whole body and tissue specific glucose uptake. (A) Whole body glucose uptake (M value), endogenous glucose production, and whole body glucose disposal rate in the carriers (red bars, N=20) and non-carriers (blue bars, N=25) of p.P50T/*AKT2*. (B, C) Tissue-specific glucose uptake in the carriers (red bars, N=20) and non-carriers (blue bars, N=25) of p.P50T/*AKT2*. Bar heights represent sample means, vertical lines represent sample SDs. *P* values for comparison of carriers vs non-carriers of p.P50T/*AKT2*. EGP, endogenous glucose production.

Figure 2. (A) Brain regions in the PET study where insulin-stimulated glucose uptake was measured in carriers and non-carriers of the p.P50T/*AKT2* variant. (B) Significant differences (*P* value) in glucose uptake in the specific regions of the brain between non-carriers (blue bars, N=25) and carriers (red bars, N=20) of the p.P50T/*AKT2* variant. Data are means ± SD.

Figure 3. (A) Correlations of the whole body glucose uptake with the tissue-specific glucose uptake in skeletal muscle, heart muscle, brown fat, subcutaneous fat, bone marrow, brain, jejunum, and liver in carriers and non-carriers of p.P50T/*AKT2* variant. (B) Correlations of the mean brain glucose uptake with tissue-specific glucose uptake in skeletal muscle, heart muscle, brown fat, subcutaneous fat, bone marrow, jejunum, and liver in carriers and non-carriers of p.P50T/*AKT2* variant. Blue arrows stand for correlations in non-carriers and red color for carriers of the p.P50T/*AKT2* variant. **P*<0.05 (exact *P* values are given in the text) for correlations that were significantly different between the carriers and non-carriers of the p.P50T/*AKT2* variant.