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ARTICLE

Metabolomics uncovers the role of adipose tissue *PDXK* in adipogenesis and systemic insulin sensitivity

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Abstract

Aims/hypothesis We aimed to investigate the potential mechanisms involved in the compromised adipogenesis of visceral (VAT) vs subcutaneous adipose tissue (SAT) using comparative metabolomics. Based on the differentially identified metabolites, we focused on the relationship between the active form of vitamin B_6 (pyridoxal 5-phosphate [PLP]), known to be generated through pyridoxal kinase (PDXK), and adipogenesis.

Methods Non-targeted metabolomics analyses were performed in paired VAT and SAT ($n = 14$, discovery cohort). *PDXK* gene expression was evaluated in two validation cohorts of paired SAT and VAT samples in relation to obesity status and insulin sensitivity, and mechanistically after weight loss in vivo and in 3T3-L1 cells in vitro.

Results Comparative metabolomics showed that PLP was significantly decreased in VAT vs SAT. Concordantly, *PDXK* mRNA levels were significantly decreased in VAT vs SAT, specifically in adipocytes. The decrease was specially marked in obese individuals. *PDXK* mRNA levels showed a strong association with adipogenic, lipid-droplet-related and lipogenic genes. At a functional level, systemic insulin sensitivity positively associated with *PDXK* expression, and surgically-induced weight loss (improving insulin sensitivity) led to increased SAT *PDXK* mRNA levels in parallel with adipogenic genes. In human pre-adipocytes, *PDXK* mRNA levels increased during adipocyte differentiation and after administration of peroxisome proliferator-activated receptor-γ agonists, and decreased under inflammatory stimuli. Mechanistic studies in 3T3-L1 cells showed that PLP administration resulted in increased adipogenic mRNA markers during early adipogenesis, whereas the PLP antagonist 4-deoxypyridoxine exerted opposite effects.

Conclusions/interpretation Overall, these results support the notion that in situ production of PLP is required for physiological adipogenesis.

Keywords Adipogenesis · Insulin sensitivity · Obesity · *PDXK* · Vitamin B_6

José María Moreno-Navarrete and Mariona Jove contributed equally to this work.

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Abbreviations

4-DP	4-Deoxypyridoxine
MCM	Macrophage-conditioned medium
PCA	Principal component analysis
PDXK	Pyridoxal kinase
PLP	Pyridoxal 5′-phosphate
PLS-DA	Partial least-square discriminant analysis
PPAR	Peroxisome proliferator-activated receptor
RIP140	Receptor interacting protein 140

SAT	Subcutaneous adipose tissue
SVF	Stromal vascular fraction
VAT	Visceral adipose tissue

Introduction

Aside from evident differences in anatomical location, adipose tissue depots present in subcutaneous and omental sites are functionally and pathophysiologically different. Obesity-related complications are mainly associated with the presence and amount of omental adipose tissue, leading to the assumption of a distinct deleterious role, as demonstrated in population studies [1]. The reasons for such differences from subcutaneous tissue may arise from differential accumulation of inflammatory cells and a different lipid composition [2]. Omental adipose tissue-derived metabolites, through the anatomical connection mediated by the portal vein, could reach hepatocytes and/or interact with absorbed nutrients [3]. Similarly, metabolic activity at each location differs markedly in sensitivity to endocrine regulators of lipolysis, such as catecholamines and insulin [3]. The differences may be related to obesity-associated growth of adipose tissue, as lipidomic signatures specific to the omental location only arise in obese individuals [2]. The use of systems biology-based approaches (such as genomics, transcriptomics, proteomics and lipidomics) permits a molecular fingerprinting of these tissues. Transcriptomics revealed differentially expressed genes belonging to adipogenesis and lipid metabolism ontogenies both in vivo [4–6] and in cells in vitro [7]. This fits with a differential pattern of protein expression, with location-specific traits in proteins involved in lipid oxidation–reduction and transport [8].

In contrast to genes and proteins, metabolites provide the most direct evidence of biochemical changes and they can be easily correlated with a given phenotype. Metabolomics based on gas chromatography techniques has been previously tested in adipose tissue from differential locations [9]. Although it is robust, this technique shows a relatively low number of potential candidates when compared with liquid chromatography coupled with high-precision mass spectrometry.

In the present work we demonstrate the existence of specific metabolomic signatures of human omental and subcutaneous adipose tissue (SAT), mainly attributed to selective enrichment in important bioactive compounds. Pyridoxal 5-phosphate (PLP) was one of the most differentially detected metabolites, being significantly decreased in visceral adipose tissue (VAT) vs SAT. Since subsequent analyses showed that *PDXK* is highly expressed in human adipose tissue, we explored the possible link between *PDXK* and adipogenesis in human adipose tissue, by employing both a 3T3-L1 mouse cell line and human adipocytes, confirming the importance of PLP and *PDXK* in adipocyte differentiation.

Methods

Recruitment of donors for adipose tissue samples

Cross-sectional studies In the first and second cohort, a group of 196 tissues (101 VAT and 95 SAT) (cohort 1) from participants with normal body weight and different degrees of obesity, with BMI 20–68 kg/m^2, were analysed (for characteristics of cohort, see Table 1). This cohort included 70 paired SAT and VAT samples (eight non-obese and 62 obese participants). In a second cohort of morbidly obese (BMI >35 kg/m^2) individuals with different degrees of insulin activity (measured using hyperinsulinaemic–euglycaemic clamp [6], as detailed in the ESM Methods), 44 paired SAT and VAT samples (cohort 2) were studied (Table 1). The participants were recruited at the Endocrinology Service of the Hospital of Girona Dr Josep Trueta. All were of European descent and reported that their body weight had been stable for at least 3 months before the study. Participants were studied in the post-absorptive state. BMI was calculated as weight (in kg) divided by height (in m) squared. They had no systemic disease other than obesity and all were free of any infections in the month leading up to the study. Liver diseases (specifically tumours and hepatitis C infection) and thyroid dysfunction were specifically excluded by biochemical work-up. All participants gave written informed consent, validated and approved by the Ethics committee of the Hospital of Girona Dr Josep Trueta, after the purpose of the study was explained to them.

Samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric bypass surgery). Samples of adipose tissue were immediately transported to the laboratory (5–10 min). The handling of tissue was carried out under strictly aseptic conditions. Adipose tissue samples were washed in PBS, cut with forceps and scalpel into small pieces (100 mg) and immediately flash-frozen in liquid nitrogen before being stored at −80°C. Adipocyte and stromal vascular fraction (SVF) cells were isolated from eight SAT and eight VAT non-frozen samples. These samples were washed three or four times with PBS and suspended in an equal volume of PBS supplemented with 1% vol./vol. penicillin–streptomycin and 0.1% (wt/vol.) collagenase type I pre-warmed to 37°C. The tissue was placed in a shaking water bath at 37°C with continuous agitation for 60 min and centrifuged for 5 min at 300–500 *g* at room temperature. The supernatant fraction, containing mature adipocytes, was collected. The pellet was identified as the SVF. Isolated mature adipocytes and SVF were stored at −80°C for gene expression analysis.

Intervention study The third cohort consisted of 18 obese (BMI 43.67 ± 4.6 kg/m^2, age 49.1 ± 7.6 years [mean ± SD])

Table 1 Anthropometric and clinical characteristics of individuals in cohort 1 and cohort 2

Characteristic	Non-obese	Obese	*p* value
Cohort 1			
N, VAT/SAT	14/12	87/83	
Age, years	44.6 ± 12.1	45.4 ± 10.2	0.8
BMI, kg/m^2	26.1 ± 2.8	44.9 ± 6.7	<0.0001
Fasting glucose, mmol/l	4.77 (4.44–5.27)	5.32 (4.82–6.21)	0.03
Fasting triacylglycerols, mmol/l	1.02 (0.76–2.26)	1.19 (0.91–1.70)	0.8
HDL-cholesterol, mmol/l	1.56 (1.16–2.17)	1.34 (1.11–1.61)	0.5
VAT *PDXK*, RU	0.095 ± 0.04	0.074 ± 0.03	0.04
SAT *PDXK*, RU	0.138 ± 0.03	0.139 ± 0.05	0.9
Cohort 2			
N	44		
Age, years	47.5 ± 8.6		
BMI, kg/m^2	44.6 ± 6.8		
Fasting glucose, mmol/l	5.41 (4.99–5.94)		
M value, mmol kg^{-1}·min^{-1}	0.020 ± 0.012		
Fasting triacylglycerols, mmol/l	1.37 (0.95–1.74)		
HDL-cholesterol, mmol/l	1.18 ± 0.24		
VAT *PDXK*, RU	0.069 ± 0.03		
SAT *PDXK*, RU	0.128 ± 0.04		

Data are means ± SD or median (interquartile range)

RU, relative gene expression units

individuals of European descent, who underwent bariatric surgery (Roux-en-Y gastric bypass [RYGB]) at the Hospital of Girona Dr Josep Trueta and were part of an ongoing study [6]. Inclusion criteria were age 30–60 years, BMI ≥ 35 kg/m^2 and ability to understand the study protocol. Exclusion criteria were use of medications able to interfere with insulin's action and history of a chronic systemic disease. Samples from the SAT depot were obtained during bariatric surgery. Postoperative samples of SAT were obtained by subcutaneous biopsy at the mesogastric level 2 years after surgery. Fasting blood samples were obtained on the same day as the biopsy. All participants gave written informed consent, validated and approved by the ethical committee of the Hospital of Girona Dr Josep Trueta, after the purpose of the study was explained to them.

Analytical methods

Serum glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyser II (Beckman Instruments, Brea, CA, USA). A Roche Hitachi Cobas c711 instrument (Roche, Barcelona, Spain) was used to determine HDL-cholesterol and total serum triacylglycerols. HDL-cholesterol was quantified by a homogeneous enzymatic colorimetric assay through the cholesterol esterase–cholesterol oxidase–peroxidase reaction (Cobas HDLC3). Serum fasting triacylglycerols were measured by an enzymatic, colorimetric method with glycerol phosphate oxidase and peroxidase (Cobas TRIGL).

Non-targeted metabolomics

Metabolites were analysed in the discovery cohort in 14 paired VAT and SAT samples (four men and ten women) with methanol according to previously described methods [2]. Briefly, 20 volumes (vol./wt) of cold methanol (containing 1 μmol/l butylated hydroxytoluene) were added to 35–50 mg of adipose tissue and homogenised with a Polytron device at 4°C. Then, 20 volumes of lysis buffer (consisting of 180 mmol/l KCl, 5 mmol/l EDTA and 1 mmol/l diethylenetriaminepentaacetic acid, pH 7.3) were added and homogenates were incubated at −20°C for 1 h and centrifuged at 12,000 *g* for 3 min. The supernatant fractions were recovered, evaporated using a Speed Vac (Thermo Fisher Scientific, Barcelona, Spain) and resuspended in 0.4% vol./vol. acetic acid and methanol (1 : 1 vol./vol.).

For the metabolomics studies, an Agilent 1290 LC system coupled to an electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) MS/MS 6520 instrument (Agilent Technologies, Barcelona, Spain) was used. In all cases, 2 μl of extracted sample was applied onto a reverse-phase column (Zorbax SB-Aq, 1.8 μm 2.1 × 50 mm; Agilent Technologies) equipped with a precolumn (Zorba-SB-C8 Rapid Resolution Cartridge, 2.1 × 30 mm 3.5 μm; Agilent Technologies) with a

column temperature of 60°C. The flow rate was 0.6 ml/min. Solvent A was composed of water containing 0.2% vol./vol. acetic acid and solvent B was composed of methanol and 0.2% acetic acid. The gradient started at 2% vol./vol. B and increased to 98% vol./vol. B in 13 min, and then held at 98% B for 6 min. Post-time was established in 5 min.

Data were collected in positive and negative electrospray mode TOF operated in full-scan mode at 50–1,600 m/z in an extended dynamic range (2 GHz), using N_2 as the nebuliser gas (10 l/min, 350°C). The capillary voltage was 4,000 V with a scan rate of 1.5 scan/s. The ESI source used a separate nebuliser for the continuous, low-level (10 l/min) introduction of reference mass compounds: 121.050873, 922.009798 (positive ion mode) and 119.036320, 966.000725 (negative ion mode), which were used for continuous, online mass calibration. The MassHunter Data Analysis Software (Agilent Technologies) was used to collect the results and the MassHunter Qualitative Analysis Software (Agilent Technologies) was used to obtain the molecular features of the samples, representing different, co-migrating ionic species of a given molecular entity using the Molecular Feature Extractor algorithm (Agilent Technologies), as described [10, 11]. Finally, the MassHunter Mass Profiler Professional Software (Agilent Technologies) was used to perform a non-targeted metabolomic analysis of the extracted features. We selected samples with a minimum absolute abundance of 5,000 counts and with a minimum of two ions. Multiple charge states were not considered. Compounds from different samples were aligned using a retention time (RT) window of 0.1% ± 0.15 min and a mass window of 5.0 ppm ± 2.0 mDa. Only common features (found in at least 75% of the samples at the same condition) were analysed, correcting for individual bias. Data for principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA) were obtained using this software. The masses with significant differences (paired *t* test, $p < 0.05$; Benjamini Hochberg correction for false discovery rate) in abundance were searched against METLIN Metabolite PCD/PCDL database (Agilent Technologies). This database allows the identification of metabolites based on isotope distribution, exact mass and identical chromatographic conditions with authentic standards. Pathway analysis was performed using Metaboanalyst platform [12] and resulting pathways were included in the ConsensusPathDB Interaction database [13].

RNA expression

RNA purification, gene expression procedures and analyses were performed, as detailed in electronic supplementary material (ESM) Methods and previously described [6, 14, 15].

Differentiation of human pre-adipocytes

Isolated human subcutaneous and visceral pre-adipocytes (Zen-Bio, Research Triangle Park, NC, USA) were cultured and differentiated as detailed in ESM Methods.

TNFα (100 ng/ml) and macrophage-conditioned medium (MCM, 5% vol./vol.) from THP-1 cells previously treated with lipopolysaccharide (10 ng/ml, 24 h) were added during both SAT and VAT adipocyte differentiation. Otherwise, after adipocyte differentiation (on day 14), SAT fully differentiated adipocytes were incubated with fresh medium (control) or fresh medium containing rosiglitazone (0.1 and 1 μmol/l). After 48 h cells were harvested and pellets and supernatant fractions were stored at −80°C.

All in vitro experiments using human cells were performed in triplicate and experimenters were blind to outcome assessment.

Mouse 3T3-L1 cell differentiation and mechanistic studies

The embryonic fibroblast mouse cell line 3T3-L1 (American Type Culture Collection; LGC Standards, Barcelona, Spain) was cultured in DMEM containing 20 mmol/l glucose, 10% vol./vol. calf bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Mycoplasma contamination was not detected in this cell line. During 3T3-L1 adipocyte differentiation, calf bovine serum was replaced with fetal bovine serum. Two days after confluence, insulin (5 μg/ml), dexamethasone (0.5 μmol/l) and isobutylmethylxanthine (0.5 mmol/l) mixture was added for 2 days, followed by 5 days with insulin (5 μg/ml) alone, which was replaced on day 5. Cells were then considered mature adipocytes, harvested on day 7 and stored at −80°C for RNA purification. To demonstrate the functional role of PLP, 4-deoxypyridoxine (4-DP; 5 and 50 μmol/l) and PLP (0.5, 5 and 50 μmol/l) administration was performed during adipocyte differentiation. These experiments were performed in triplicate and experimenters were blind to outcome assessment.

Statistical analyses

Statistical analyses were performed using SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean ± SD for Gaussian variables or median and interquartile range. Variables that did not fulfil normal distribution were log transformed to improve symmetry for subsequent analyses. The relationship between variables was analysed by simple correlation (Pearson's test and Spearman's test) and multiple regression analyses in a stepwise manner. For non-targeted metabolomics, a paired *t* test was used, with Benjamini–Hochberg correction for false discovery rate. Otherwise, one-factor ANOVA with post hoc Bonferroni test, paired *t* test and unpaired *t* test were used to

compare *PDXK* gene expression according to obesity. A non-parametric test (Mann–Whitney test) was used to analyse in vitro experiments. In all cases, the level of statistical significance was set at $p < 0.05$.

Results

Non-targeted metabolomics discloses a location-specific metabolomics profile

Non-targeted metabolomics showed that many molecular features (51 downregulated and 513 upregulated in VAT vs SAT, in positive ionisation; 37 downregulated and 98 upregulated in VAT vs SAT in negative ionisation) differed significantly in their concentrations (ESM Dataset 1), as demonstrated by hierarchical clustering, heatmaps and both unsupervised (PCA) and supervised multivariate analyses (PLS-DA) with an accuracy exceeding 95% (Fig. 1a, b). To reinforce this, we employed alternative classification procedures, such as random forest analyses, which demonstrated an error rate below 18% (Fig. 1c). Furthermore, the recursive supported vector machine scheme showed a model with a 7.1% error rate with only five molecular species (Fig. 1d). Multivariate classification procedures indicate a specific profile of metabolites in SAT vs VAT. As indicate both by Random forest analyses (Fig. 1c) and recursive supported vector machine classification (Fig. 1d), a relatively low error of classification could be obtained with few compounds.

Based on accurate mass, isotope distribution and retention time equality with authentic standards, nine metabolites were identified (Table 2). Pathway analyses (ESM Table 1 and ESM Fig. 1) clustered these metabolites across two major networks: one comprising adipocytokine and peroxisome proliferator-activated receptor (PPAR) signalling and the other including vitamin metabolism. Based on very early data [16–19] we focused on vitamin B_6 metabolism: the levels of vitamin B_6 active metabolite PLP were significantly decreased in VAT vs SAT (Fig. 2a). Consistently, in the discovery and validation cohorts, mRNA levels of *PDXK*, encoding the enzyme needed for PLP synthesis, were also detected in human adipose tissue, being significantly downregulated in VAT vs SAT (Fig. 2b, c). Interestingly, *PDXK* gene expression was positively correlated with PLP levels in SAT (Fig. 2d), but not in VAT (Fig. 2e). *PDXK* mRNA was significantly enriched in the adipocyte fraction of adipose tissue (Fig. 2f).

PDXK is implicated in adipogenic differentiation in human adipose tissue

To test whether PDXK could be implied in the adipogenic program of human adipose tissue, we compared its expression with accepted markers of adipogenesis in three independent cohorts. In the discovery cohort (Table 1), *PDXK* gene expression was significantly associated with expression of adipogenic genes, including *PPARG*, lipid-droplet-related (*CIDEC*, *PLIN1*) and lipogenesis-related (*FASN*, *ACACA*) genes both in SAT and in VAT (Table 3). Otherwise, *PDXK* gene expression was negatively associated with *TMEM26* mRNA levels (a specific marker of beige adipocytes) and no significant relationships were found with the expression of inflammatory gene markers (*LEP*, *IL6*, *TNF* and *CD68*) (Table 3). VAT *PDXK* gene expression was especially decreased in obese participants (Table 1) and negatively associated with BMI (Table 3). These results were confirmed in a validation cohort (Table 1) in which *PDXK* gene expression was negatively correlated with BMI and *TMEM26* in VAT, and positively associated with adipogenic (*PPARG*, *ADIPOQ*, *GLUT4* [also known as *SLC2A4*]), lipid-droplet-related (*CIDEC, PLIN1*) and lipogenic (*DGAT1*) gene expression in both VAT and SAT (Table 4).

To confirm these associations in paired VAT and SAT, all paired samples from both discovery and validation cohorts ($n = 114$) were analysed together. Interestingly, in both VAT and SAT, *PDXK* gene expression was positively correlated with the expression of adipogenic markers, including *ADIPOQ* ($r = 0.31$, $p = 0.001$ in VAT and $r = 0.33$, $p = 0.001$ in SAT), *GLUT4* ($r = 0.44$, $p < 0.0001$ in VAT and $r = 0.50$, $p < 0.0001$ in SAT), *PLIN1* ($r = 0.40$, $p < 0.0001$ in VAT and $r = 0.43$, $p < 0.0001$ in SAT) and *CIDEC* ($r = 0.46$, $p < 0.0001$ in VAT and $r = 0.51$, $p < 0.0001$ in SAT), and negatively with *TMEM26* ($r = -0.29$, $p = 0.004$ in VAT and $r = -0.28$, $p = 0.004$ in SAT).

To further study the potential importance of PLP in adipogenesis at a functional level, SAT *PDXK* gene expression was studied according to systemic insulin sensitivity (measured using hyperinsulinaemic–euglycaemic clamp), confirming a positive association (Table 4). Bariatric surgery-induced weight loss, which resulted in a significant increase in the expression of adipogenic genes [6], also led to increased *PDXK* mRNA levels gene expression (Table 5). Of note, the change in *PDXK* gene expression was positively correlated with the change in adipogenic gene expression (*FASN* [$r = 0.67$, $p = 0.004$], *PLIN1* [$r = 0.71$, $p = 0.002$], *DGAT1* [$r = 0.74$, $p = 0.001$], *IRS1* [$r = 0.58$, $p = 0.01$], *PPARG* [$r = 0.47$, $p = 0.06$] and *ADIPOQ* [$r = 0.44$, $p = 0.09$]).

PDXK is required for full adipogenic differentiation

PDXK gene expression increased during adipocyte differentiation of both VAT and SAT adipocytes, with the increase being significantly attenuated in VAT vs SAT adipocyte differentiation (Fig. 2g, h). This is in line with decreased PLP values in VAT in vivo, suggesting a differential response towards the adipogenic effects of vitamin B_6 according to anatomical location. Inflammatory stimuli resulted in decreased *PDXK*

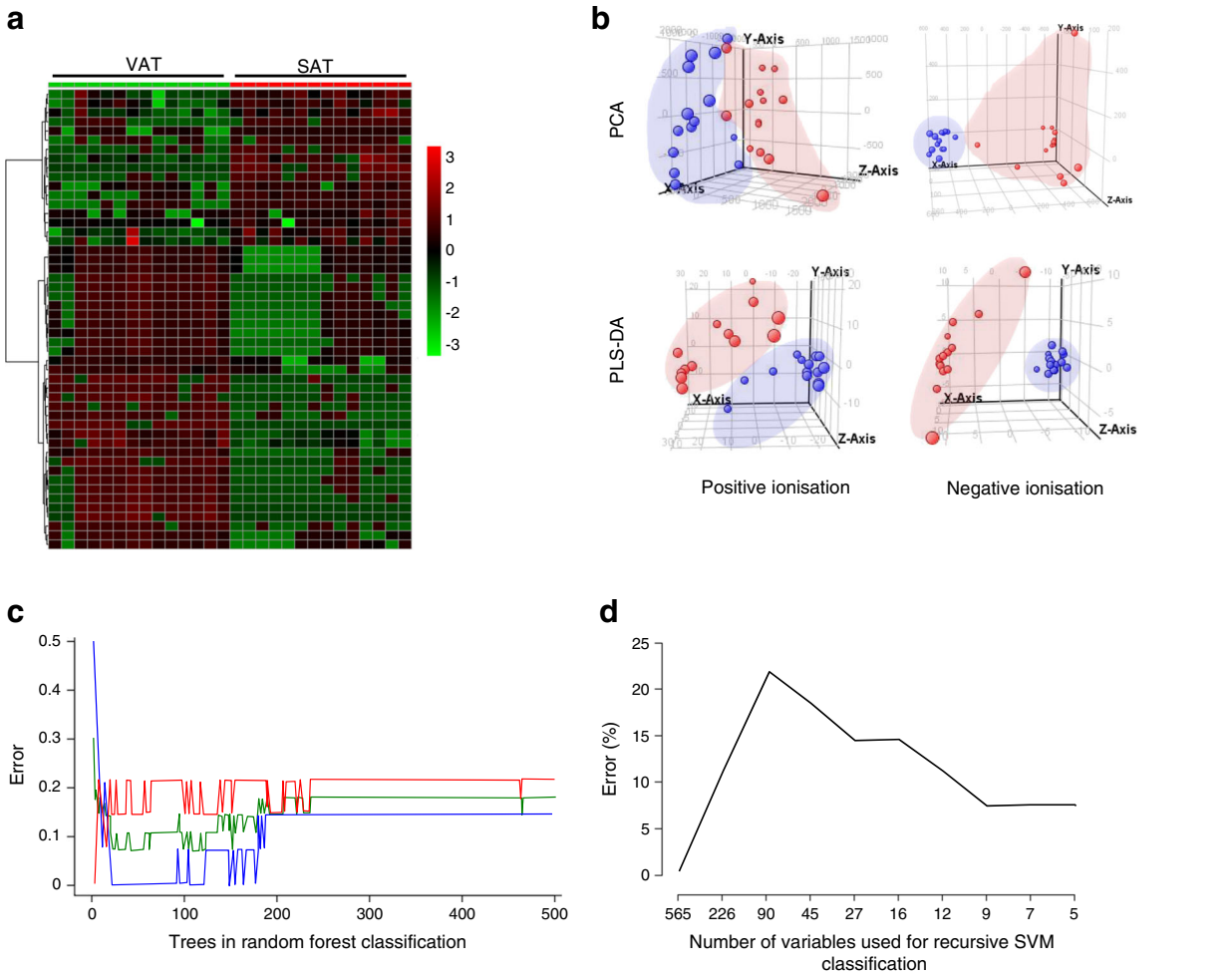

Fig. 1 (**a**, **b**) Location-specific metabolomics profiling discloses PLP metabolism as a differential trait with both hierarchical clustering of samples (Euclidean distances, Ward clustering algorithm), using the top 50 differentially expressed molecules selected by random forest algorithm (colour range bar shows the concentration of these differential molecules [log range for autoscaled data]) (**a**) and multivariate analyses (PCA and PLS-DA) (**b**), with red spheres indicating VAT samples and blue spheres SAT samples. (**c**, **d**) Multivariate classification procedures performed included random forest analyses (**c**) and recursive supported vector machine (SVM) classification (**d**). For random forest classification, an overall classification error of 0.17 (in green) was obtained (0.14 for SAT, in blue; 0.21 for VAT, in red)

gene expression (Fig. 2i), whereas PPARγ agonists led to increased *PDXK* mRNA levels in a dose-dependent manner (Fig. 2j). In line with data from human samples, and reinforcing the importance of PLP in adipogenesis, PLP

Table 2 Metabolites with significantly different concentrations between VAT and SAT

Compound	ID (HMDB database)	ID (CAS)	Change (VAT vs SAT)	*p* value[a]
Deoxycytidine	HMDB00014	951-77-9	Down	0.003421951
Proline	HMDB00162	147-85-3	Up	0.012944922
Guaiacol	HMDB01398	90-05-1	Up	0.004879887
2-Aminohexadecanoic acid	NI[1]	7769-79-1	Up	0.001562683
5α-Androstane-3,17-dione	HMDB11608	846-46-8	Up	0.006690529
Farnesal	HMDB60356	19317-11-4	Up	0.002234416
Retinoic acid	HMDB02369	302-79-4	Down	0.032992125
3-Methylindole	HMDB00466	83-34-1	Up	0.00004325
PLP	HMDB01491	54-47-7	Down	0.00037100

[a] Paired *t* test, at least $p < 0.05$ corrected for false discovery rate with Benjamini–Hochberg method

NI, not indexed

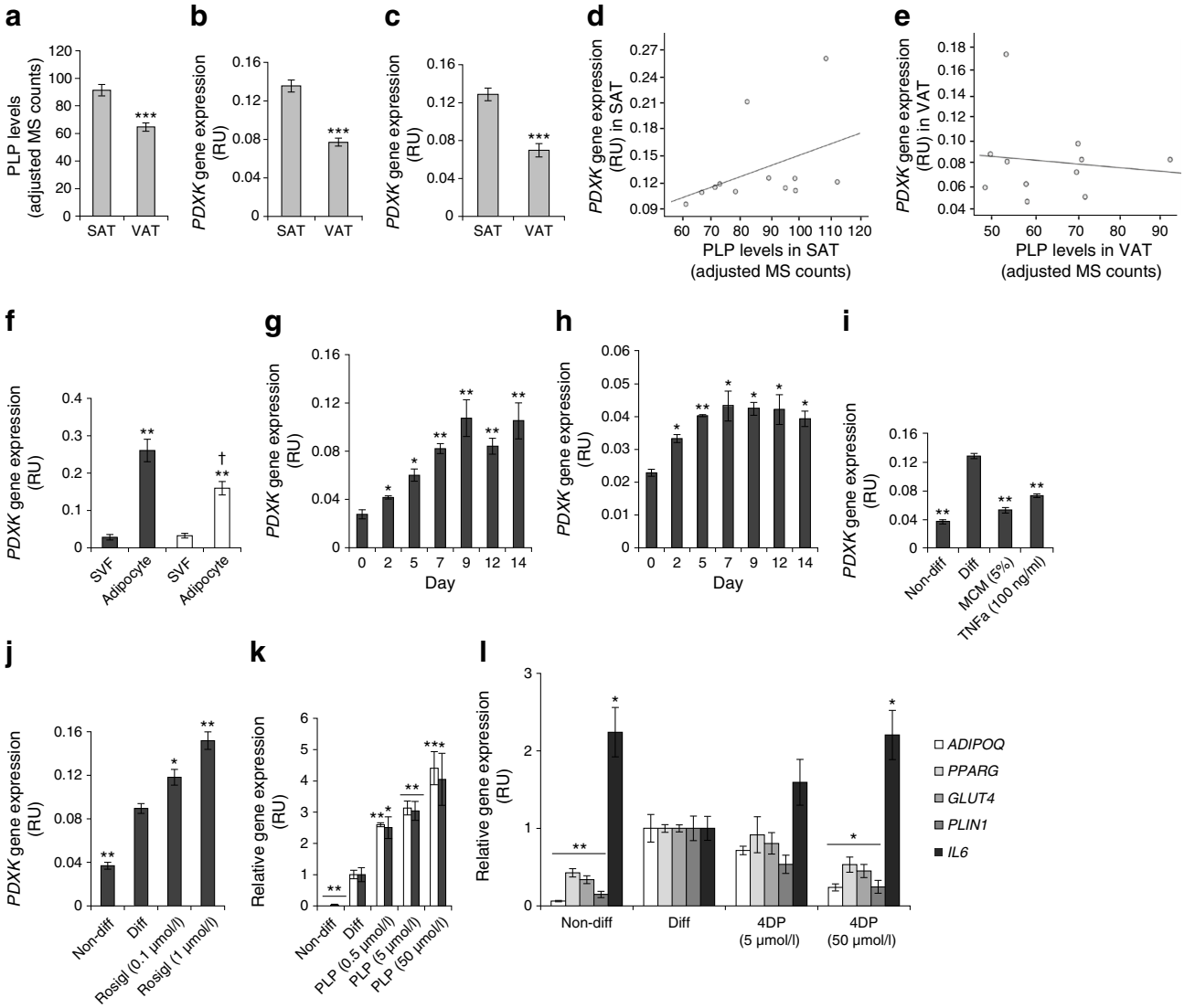

Fig. 2 (**a**–**c**) PLP levels were diminished in VAT ($n = 14$) (**a**), shown in adjusted mass spectrometry (MS) counts, and this was associated with diminished expression of *PDXK* in VAT in two different cohorts (**b**, **c**): cohort 1 ($n = 70$) (**b**) and cohort 2 ($n = 44$) (**c**). ***$p < 0.001$ compared with SAT. (**d**, **e**) Furthermore, SAT *PDXK* ($r = 0.58$, $p = 0.04$) (**d**) but not VAT ($r = -0.07$, $p = 0.8$) (**e**) expression correlates with PLP concentrations. Although PLP levels were analysed in all samples, mRNA was available in 12 of these 14 paired samples. Furthermore, PLP levels were very low in one of the VAT samples. (**f**) *PDXK* gene expression in adipose tissue fractions in SAT (dark grey bars) and VAT (light grey bars). **$p < 0.01$ compared with SVF; $^{\dagger}p < 0.05$ compared with subcutaneous adipocytes. (**g**, **h**) *PDXK* expression during subcutaneous (**g**) and visceral (**h**) adipocyte differentiation. *$p < 0.05$ and **$p < 0.01$ compared with day 0. (**i**) Effect of inflammatory stimuli on *PDXK* gene expression during subcutaneous adipocyte differentiation. **$p < 0.01$ compared with differentiated control (Diff). (**j**) Effect of exposure to a PPAR agonist (rosiglitazone, Rosigl; 0.1 and 1 µmol/l) on *PDXK* gene expression in mature adipocytes. *$p < 0.05$ and **$p < 0.01$ compared with differentiated control (Diff). (**k**) Effect of PLP (0.5, 5 and 50 µmol/l) administration on adipogenic gene expression, such as *ADIPOQ* (white bars) and *GLUT4* (dark grey bars). (**l**) Effect of 4DP (5 and 50 µmol/l) administration on expression of adipogenesis transcriptional markers, such as *ADIPOQ*, *PPARG*, *GLUT4*, *PLIN1*, and the inflammatory *IL6* gene. *$p < 0.05$ and **$p < 0.01$ compared with differentiated control (Diff)

administration (0.5, 5 and 50 µmol/l) during early adipogenesis (day 0–2) resulted in a dose-dependent increase in adipogenic gene expression markers (*ADIPOQ* and *GLUT4*) (Fig. 2k). On the contrary, administration of 4DP, a PLP antagonist (50 µmol/l), led to a significant reduction in the expression of adipogenic genes (*ADIPOQ*, *PPARG*, *GLUT4* and *PLIN1*) and increased expression of the proinflammatory gene *IL6* (Fig. 2l).

Discussion

Pyridoxal (pyridoxine, vitamin B_6) kinase is a cytosolic enzyme (EC:2.7.1.35) that phosphorylates vitamin B_6, an essential step for the conversion of vitamin B_6 to PLP (the active form of vitamin B_6) [20]. PLP acts as a cofactor for more than 140 enzymes, which catalyse multiple biochemical reactions and participate in a wide range of

Table 3 Correlation analysis of the association between *PDXK* gene expression and anthropometric and clinical characteristics and selected mRNAs in SAT and VAT from cohort 1, in all participants studied and in paired adipose tissue samples

Characteristic	VAT		SAT	
	r	*p* value	*r*	*p* value
Cohort 1—all samples[a]				
Age (years)	0.26	0.01	0.14	0.2
BMI (kg/m^2)	−0.24	0.03	0.05	0.6
Fasting glucose (mmol/l)	0.05	0.6	−0.01	0.9
Fasting triacylglycerols (mmol/l)	0.01	0.9	0.07	0.5
HDL-cholesterol (mmol/l)	0.08	0.5	−0.01	0.9
PPARG (RU)	0.49	<0.0001	0.32	0.01
CIDEC (RU)	0.31	0.01	0.54	<0.0001
PLIN1 (RU)	0.47	<0.0001	0.44	<0.0001
FASN (RU)	0.26	0.01	0.25	0.02
ACACA (RU)	0.25	0.01	0.27	0.01
TMEM26 (RU)	−0.41	<0.0001	−0.31	0.01
LEP (RU)	−0.07	0.7	0.21	0.1
IL6 (RU)	−0.01	0.9	0.07	0.5
TNF (RU)	0.02	0.8	−0.04	0.7
CD68 (RU)	−0.06	0.6	−0.23	0.06
Cohort 1—paired samples[b]				
Age (years)	0.10	0.4	0.07	0.5
BMI (kg/m^2)	−0.23	0.05	0.07	0.5
Fasting glucose (mmol/l)	−0.08	0.5	−0.03	0.8
Fasting triacylglycerols (mmol/l)	−0.22	0.06	0.14	0.2
HDL-cholesterol (mmol/l)	0.20	0.09	−0.08	0.5
PPARG (RU)	0.51	<0.0001	0.21	0.07
CIDEC (RU)	0.41	0.001	0.55	<0.0001
PLIN1 (RU)	0.27	0.03	0.42	0.001
FASN (RU)	0.34	0.005	0.23	0.05
ACACA (RU)	0.32	0.01	0.21	0.07
TMEM26 (RU)	−0.31	0.01	−0.37	0.002
LEP (RU)	−0.04	0.7	0.19	0.1
IL6 (RU)	0.02	0.8	0.12	0.3
TNF (RU)	0.12	0.3	−0.10	0.4
CD68 (RU)	−0.16	0.2	−0.22	0.06

[a] $n = 101$ for VAT and 95 for SAT

[b] $n = 70$ for VAT and 70 for SAT

RU, relative gene expression units

Table 4 Correlation analysis of the association between *PDXK* gene expression and anthropometric and clinical characteristics and selected mRNAs in SAT and VAT from cohort 2

Characteristic	VAT ($n = 44$)		SAT ($n = 44$)	
	r	*p* value	*r*	*p* value
Age (years)	0.24	0.1	0.08	0.5
BMI (kg/m^2)	−0.47	0.002	0.09	0.5
Fasting glucose (mmol/l)	0.06	0.7	0.01	0.9
M value (mmol kg^{-1}·min^{-1})	0.19	0.2	0.40	0.01
Fasting triacylglycerols (mmol/l)	−0.08	0.6	−0.02	0.8
HDL-cholesterol (mmol/l)	0.11	0.4	0.24	0.1
PPARG (RU)	0.56	<0.0001	0.09	0.5
CIDEC (RU)	0.77	<0.0001	0.49	0.001
PLIN1 (RU)	0.74	<0.0001	0.43	0.005
ADIPOQ (RU)	0.41	0.01	0.36	0.01
GLUT4 (RU)	0.59	<0.0001	0.46	0.001
DGAT1 (RU)	0.61	<0.0001	0.63	<0.0001
TMEM26 (RU)	−0.49	0.004	0.03	0.8
IL6 (RU)	−0.18	0.3	-0.22	0.2
TNF (RU)	0.09	0.6	0.21	0.2

RU, relative gene expression units

metabolic and developmental processes including amino acid, fatty acid and neurotransmitter metabolism [20, 21]. Furthermore, PLP attenuates the production of oxygen reactive species [22] and the formation of advanced glycation end-products [23]. In fact, vitamin B has been used in the treatment of diabetic complications [24], though its use has to be viewed with caution [25].

To the best of our knowledge, this is the first study to demonstrate substantial PLP levels and *PDXK* gene expression in human adipose tissue, with evident differences between pathogenically relevant distinct anatomical locations. The link between PDXK and adipogenesis was supported by several lines of evidence. First, in line with adipogenic markers [6, 26], PLP levels and *PDXK* gene expression was decreased in VAT vs SAT and adipocytes, with the associated differences in their adipogenic potential. Second, *PDXK* gene expression was significantly associated with the expression of adipogenic genes in the discovery and validation cohort. Similar to lipogenic and adipogenic genes [6, 26], VAT *PDXK* mRNA levels were negatively correlated with BMI. Third, bariatric surgery-induced weight loss led to increased *PDXK* gene expression in positive correlation with adipogenic genes. Fourth, similar to adipogenic genes, *PDXK* mRNA levels increased during human adipocyte differentiation. Inflammatory conditions attenuated the increase of *PDXK* mRNA levels during adipocyte differentiation, whereas a PPARγ agonist enhanced *PDXK* mRNA levels. Finally, the inhibition of PDXK activity (using 4-DP) attenuated adipogenic gene expression during adipocyte differentiation, whereas PLP administration in the early stage of adipocyte differentiation had the opposite effect.

Altogether these data support the notion that in situ production of PLP is required for physiological adipogenesis. Previous knowledge was based on the effects of vitamin B_6 per se. A vitamin B_6-deficient diet resulted in a significant

Table 5 Effects of bariatric surgery-induced weight loss on *PDXK* gene expression, anthropometric and clinical characteristics and biochemical variables in cohort 3 ($n = 18$)

Characteristic/variable	Baseline	Post-weight loss	*p* value
Age (years)	49.1 ± 7.6		
BMI (kg/m^2)	43.67 ± 4.6	29.32 ± 5.17	<0.0001
Fasting glucose (mmol/l)	5.68 ± 2.10	4.88 ± 0.87	0.04
M value (mmol kg^{-1}·min^{-1})	0.023 ± 0.013	0.033 ± 0.011	0.001
HDL-cholesterol (mmol/l)	1.45 ± 0.33	1.87 ± 0.54	<0.0001
Fasting triacylglycerols (mmol/l)	1.33 ± 0.59	1.01 ± 0.53	0.001
C-reactive protein (nmol/l)	90.91 ± 7.69	8.45 ± 5.22	<0.0001
PDXK gene expression (RU)	0.112 ± 0.037	0.151 ± 0.044	0.001
ADIPOQ gene expression (RU)	3.43 ± 1.32	4.58 ± 1.23	0.01
IRS1 gene expression (RU)	0.008 ± 0.004	0.014 ± 0.005	0.01

Data are means ± SD. RU, relative gene expression units

reduction in adipose tissue and lipogenesis in rat models [16–18]. Vitamin B_6 administration increased intracellular lipid accumulation in 3T3-L1 adipocytes [27] and reduced macrophage infiltration and adipose tissue inflammation in mice [19, 28].

Current findings show that vitamin B_6 needs to be transformed to PLP to show these effects. In fact, PLP induced PPARγ target genes in 3T3-L1 and NHI3T3 cells, stimulating PPARγ transactivation [27]. PPARγ binding sites are co-localised in the promoters of most genes, which change during adipogenesis [29–31]. In the current study, PPAR signalling appeared as a relevant node in the metabolomic network analyses differentiating SAT and VAT. However, other hypotheses on the role of PLP in adipogenesis cannot be dismissed. Huq et al [32] demonstrated that mouse nuclear receptor interacting protein 140 (RIP140) can be modified by PLP conjugation and, in consequence, RIP140 transcriptional co-repressive activity and its physiological function in adipocyte differentiation are enhanced. RIP140 participates in adipocyte fat accumulation and lipogenesis and in the maintenance of white adipocyte phenotype [33, 34]. Otherwise, PLP is a cofactor required for the optimal activity of both mitochondrial and cytoplasmic isoenzymes of serine hydroxymethyltransferase (SHMT1) and (SHMT2) [35, 36]. These enzymes are essential in folate-mediated one-carbon metabolism, which is compartmentalised in the mitochondria and cytoplasm and is known to have an important role in DNA methylation, synthesis and repair [36, 37]. Altered DNA methylation pattern has been recently associated with adipose tissue dysfunction in patients with type 2 diabetes [38]. Current data showing a link between PLP levels and adipogenesis imply that PLP has a possible role in adipose tissue DNA methylation through folate-mediated one-carbon metabolism. In fact, deficiency of vitamin B_6 and B_{12} is known to be associated with altered methylation pattern and decreased DNA stability through inhibition of DNA synthesis and repair [39]. PLP has an important role in the maintenance of genome integrity [40]. Further studies will be required to explore this hypothesis.

Another potentially important finding of the present study was the positive association between SAT *PDXK* gene expression and insulin sensitivity. Furthermore, *PDXK* mRNA levels after bariatric surgery-induced weight loss ran in parallel with increased systemic insulin sensitivity in association with the expression of PPARγ target genes [27, 41–43]. In line with these results, plasma PLP levels were found to be negatively associated with insulin resistance-associated variables (such as C-reactive protein concentration, fasting glucose and HbA_{1c}) in a previous study [44], whereas increased plasma PLP levels correlated with blood glucose reduction in surgically ill patients [45].

In conclusion, the present study suggests a novel role of adipose tissue PDXK activity and PLP levels in adipogenesis and systemic insulin sensitivity, with anatomical differences between SAT and VAT.

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