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# Pioglitazone corrects dysregulation of skeletal muscle mitochondrial proteins involved in ATP synthesis in type 2 diabetes

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**Abstract**

**Context:** In this study, we aimed to identify the determinants of mitochondrial dysfunction in skeletal muscle (SKLM) of subjects with type 2 diabetes (T2DM), and to evaluate the effect of pioglitazone (PIO) on SKLM mitochondrial proteome.

**Methods:** Two different groups of adults were studied. Group I consisted of 8 individuals with normal glucose tolerance (NGT) and 8 with T2DM, subjected to SKLM mitochondrial proteome analysis by 2D-gel electrophoresis followed by mass spectrometry-based protein identification. Group II included 24 individuals with NGT and 24 with T2DM, whose SKLM biopsies were subjected to immunoblot analysis. Of the 24 subjects with T2DM, 20 were randomized to receive placebo or PIO (15 mg daily) for 6 months. After 6 months of treatment, SKLM biopsy was repeated.

**Results:** Mitochondrial proteomic analysis on Group I revealed that several mitochondrial proteins involved in oxidative metabolism were differentially expressed between T2DM and NGT groups, with a downregulation of ATP synthase alpha chain (ATP5A), electron transfer flavoprotein alpha-subunit (ETFA), cytochrome c oxidase subunit VIb isoform 1 (CX6B1), pyruvate dehydrogenase protein X component (ODPX), dihydrolipoamide dehydrogenase (DLDH), dihydrolipoamide-S-succinyltransferase (DLST), and mitofilin, and an up-regulation of hydroxyacyl-CoA-dehydrogenase (HCDH), 3,2-trans-enoyl-CoA-isomerase (D3D2) and delta3,5-delta2,4-dienoyl-CoA-isomerase (ECH1) in T2DM as compared to NGT subjects. By immunoblot analysis on SKLM lysates obtained from Group II we confirmed that, in comparison to NGT subjects, those with T2DM exhibited lower protein levels of ATP5A (-30%, $P$=0.006), ETFA (-50%, $P$=0.02), CX6B1 (-30%, $P$=0.03), key factors for ATP biosynthesis, and of the structural protein mitofilin (-30%, $P$=0.01). T2DM was associated with a reduced abundance of the enzymes involved in the Krebs cycle DLST and ODPX (-20%, $P \leq 0.05$) and increased levels of HCDH and ECH1, enzymes implicated in the fatty acid catabolism (+30%, $P \leq 0.05$).

In subjects with type 2 diabetes treated with PIO for 6 months we found a restored SKLM protein abundance of ATP5A, ETFA, CX6B1, and mitofilin. Moreover, protein levels of HCDH and ECH1 were reduced by -10% and -15% respectively ($P \leq 0.05$ for both) after PIO treatment.

**Conclusion:** Type 2 diabetes is associated with reduced levels of mitochondrial proteins involved in oxidative phosphorylation and an increased abundance of enzymes implicated in fatty acid catabolism in SKLM. PIO treatment is able to improve SKLM mitochondrial proteomic profile in subjects with T2DM.

*Trial registration*: Clinical.Trial.gov NCT01223196

**Keywords:** Mitochondrial proteomics; type 2 diabetes, human skeletal muscle biopsies; mitochondrial dysfunction; pioglitazone.

**Abbreviations:**

ATPA: ATP synthase alpha chain
CX6B1: cytochrome c oxidase subunit VIb isoform 1
DLDH: dihydrolipoamide dehydrogenase
DLST: dihydrolipoamide-S-succinyltransferase
D3D2: 3,2-trans-enoyl-CoA-isomerase
ECH1: delta3,5-delta2,4-dienoyl-CoA-isomerase
ETFA: electron transfer flavoprotein alpha-subunit
HCDH: hydroxyacyl-CoA-dehydrogenase
HDL: high lipoprotein density
IPG: immobilized pH gradient
LDL: low density lipoprotein
NGT: normal glucose tolerance
ODPX: pyruvate dehydrogenase protein X component
PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1α
ROS: reactive oxygen species

## 1. Introduction

Insulin resistance in skeletal muscle represents a major etiologic factor in the pathogenesis of type 2 diabetes [1-2]. The mechanisms underlying the impaired insulin responsiveness of insulin target tissues are complex and still incompletely understood. An increasing body of evidence has accumulated indicating a link between mitochondrial dysfunction and insulin resistance [3-6]. In skeletal muscle, mitochondria represent the main source of energy obtained through the enzymatic oxidation of carbohydrates and lipids, and mitochondrial ATP production is essential for the functional and structural integrity of skeletal muscle cells. Cumulative evidence indicates that type 2 diabetes is associated with reduced muscle oxidative capacity and impaired mitochondrial function [4-9], which inhibit insulin signaling through different mechanisms [4,6,10-12]. We and others have shown that disturbances in glucose and fatty acid metabolism result in intracellular accumulation of fatty acid and glycolytic intermediates, which in turn activate molecular pathways such as protein kinase C, nuclear factor-κB, and toll-like receptor 4 networks, known to interfere with insulin signaling [10-16]. Additionally, ATP depletion due to the reduced oxidative phosphorylation has been shown to contribute to insulin resistance by compromising catalytic activity of numerous enzymes involved in insulin signal transduction [17,18]. In addition to energy production, mitochondria are a major source of reactive oxygen species (ROS), and an increased intracellular burden of ROS due to mitochondrial dysfunction is a well-known contributor to insulin resistance in skeletal muscle [11,19-20]. On the other hand, insulin resistance and hyperglycemia may induce mitochondrial dysfunction leading to a negative feedback circle that impairs glucose homeostasis [11,21].

Previous microarray studies designed to examine gene expression abnormalities in skeletal muscle in type 2 diabetic patients have revealed a pattern of reduced expression of genes involved in mitochondrial function [22-24]. However, changes in mRNA levels may not be mirrored by

changes in protein abundance. This limitation of these studies supports the need of concomitant proteomic profile analyses. Utilizing proteomic approaches, a number of abnormalities in the skeletal muscle proteome of type 2 diabetic subjects have been described, including a reduced abundance of several mitochondrial proteins involved in oxidative metabolism [25-28]. However, these studies were carried out in a small number of individuals and their findings require confirmation in a larger population.

The thiazolidinedione pioglitazone is an anti-diabetic agent able to improve insulin sensitivity and glycemic control through a variety of molecular mechanisms which have been only partially elucidated [29-31]. Evidence suggests that pioglitazone promotes the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a master modulator of mitochondrial biogenesis and function, thereby, improving mitochondrial oxidative metabolism [30,32-34].

In this study, we aimed to identify the determinants of mitochondrial dysfunction in skeletal muscle of subjects with type 2 diabetes by comparing the skeletal muscle mitochondrial proteomic profile of individuals with type 2 diabetes with that of subjects with normal glucose tolerance (NGT). Moreover, since pioglitazone treatment has been shown to improve mitochondrial function, we evaluated whether pioglitazone can modulate the mitochondrial proteomic pattern in the skeletal muscle of type 2 diabetic subjects.

## 2. Materials and Methods

### *2.1 Subjects*

Two groups of adult individuals were studied at the Bartter Clinical Research Unit (BRU) of the South Texas Veterans Healthcare System, University of Texas Health Science Center at San Antonio. Group I consisted of 8 individuals with NGT and 8 with type 2 diabetes. Following an overnight fasting, subjects underwent to a vastus lateralis muscle biopsy, and analysis of the skeletal muscle mitochondrial proteome by 2D-gel electrophoresis followed by mass spectrometry-based protein identification was performed on subcellular protein lysates . Group II consisted of 24 individuals with NGT and 24 with type 2 diabetes. After an overnight fasting, they were subjected to a vastus lateralis muscle biopsy, followed by immunoblot analysis of total protein lysates. Of the 24 subjects affected by type 2 diabetes, 20 were randomized to receive placebo (n=9) or pioglitazone (15 mg daily, n=11) for 6 months. After 6 months of treatment, the vastus lateralis muscle biopsy was repeated as described in a previously published study [29].

Inclusion criteria included age = 18-70 years; BMI = 24-40 kg/m2, and males/females in equal distribution. All control subjects had a normal OGTT. Other than antidiabetic medications (metformin and/or sulfonylureas), no subjects were taking any medications known to affect glucose metabolism. Statin and antihypertensive therapy was allowed if the dose was stable for at least 4 months. Body weight was stable (±3 pounds over the preceding 3 months) and no subject participated in excessively heavy exercise programs (≥30 minutes/day, ≥3 days/week, walking) [35] . Diabetic subjects taking insulin, thiazolidinediones, glucagon like peptide 1 analogues or dipeptidyl peptidase-IV inhibitors were excluded from the study.

### *2.2 Experimental Design*

Following a ~10-hour overnight fast, all subjects had a 75 g OGTT with measurement of plasma glucose and insulin concentrations at -30, -15, 0, 30, 60, 90 and 120 min.

On a separate day, following an ~10-hour overnight fast subjects underwent to a four-hour euglycemic hyperinsulinemic clamp (80 mU/m$^2$•min) as previously described [29,32]. Briefly, a priming dose of insulin (Humulin, Eli Lilly & Co., Indianapolis, IN) was administrated during the initial 10 minutes to acutely raise the plasma insulin concentration followed by a continuous insulin infusion at 80 mU/m$^2$• min. In diabetic participants, plasma glucose was allowed to decline to 100 mg/dl at which it was maintained by a variable infusion of 20% glucose. Sixty minutes prior to the start of the insulin clamp a percutaneous needle biopsy of the vastus lateralis muscle was performed in all study participants under local anaesthesia using a Bergström needle and the muscle specimens were immediately frozen in liquid nitrogen and stored at -80˚C until analyzed. A second euglycemic hyperinsulinemic clamp and percutaneous needle biopsy of the vastus lateralis muscle was performed in 20 type 2 diabetic patients 6 months after treatment with pioglitazone (n=11) or placebo (n=9).

The study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Written informed consent was obtained from all study participants in accordance with the guidelines of the Declaration of Helsinki.

### *2.3 Biochemical Analyses*

Plasma total and high lipoprotein density (HDL) cholesterol, triglycerides and glucose levels were measured by enzymatic method (GM9, Analox Instruments Ltd, UK). Plasma insulin concentration was measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA).

### *2.4 Proteomic analysis*

#### *2.4.1 Sample preparation and subcellular fractionation*

Subcellular fractionation was performed on skeletal muscle biopsies obtained from subjects in Group I using differential centrifugation to obtain mitochondrial enriched lysates as previously

described [36-38]. The entire procedure was performed at 4°C using buffers containing protease and phosphatase inhibitors (Pierce, Rockford, IL, USA). Specifically, 25 mg of skeletal muscle tissue was homogenized in a homogenization buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl pH 7.5, 10 mM EDTA) using a Dounce homogenizer. The homogenate was centrifuged at 800 rpm for 10 min at 4°C. To improve mitochondrial enrichment from the muscle lysates, the pellet containing unbroken cells was re-homogenized and centrifuged at 800 rpm for 10 min at 4°C. Supernatants were combined and centrifuged at 10,000 rpm for 10 min. The pellet was washed for 15 min in homogenization buffer for a total of 2 times. This pellet, resuspended in the homogenization buffer, represented the mitochondrial enriched fraction.

*2.4.2 Two-dimensional gel electrophoresis*

Mitochondrial enriched fractions were re-solubilized in a solution containing 8 M urea, 50 mM DTT, 2% (w/v) CHAPS, 0.2% (w/v) carrier ampholytes and 0.001% (w/v) bromophenol blue. Protein concentration was measured in this solution using the EZQ protein quantitation kit (Invitrogen, Carlsbad, CA, USA). An 11 cm linear immobilized pH gradient (IPG) Readystrip (pH 3-10) (BioRad, Hercules, CA, USA) was passively rehydrated overnight with 185 µL of IEF solution that contained 200 µg of solubilized mitochondrial enriched proteins. Isoelectric focusing was conducted at 30,000 V/h for a maximum of 5000 V using the Protean IEF cell (BioRad, Hercules, CA, USA). To prepare the sample for the second dimension electrophoresis, the IPG strip was first equilibrated for 20 min by rocking in a solution of 0.15 M BisTris, 100 mM HCl, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 5 mM TBP, and 2.5% (w/v) iodoacetaamide [37-40]. IPG strips were placed directly on top of the Criterion precast 8-16% gradient gel (BioRad, Hercules, CA, USA) and embedded with molten 1% (w/v) agarose. Gels were run in a dodeca cell at 25 V for 2 h and then at 85 V overnight at 5-8°C until complete.

*2.4.3 Gel staining, image acquisition and 2-D image analysis*

After fixation in a solution of 10% (v/v) methanol and 7% (v/v) acetic acid, gels were stained in 100 ml of SYPRO Ruby fluorescent stain (Invitrogen, Carlsbad, CA, USA) overnight. Following staining, the gels were rinsed in double deionised water and destained for 30 min in a solution containing 10% (v/v) methanol and 7% (v/v) acetic acid to reduce the background fluorescence. The gels were immediately imaged using a Molecular Imager FX (Biorad, Hercules, CA, USA) and image analysis was performed by using PDQuest 1.0 2-D imaging software (BioRad, Hercules CA, USA).

*2.4.4 Mass spectrometric protein identification*

Protein spots were excised from the gel using a Proteome Works spot cutting robot (Bio-Rad, Hercules, CA, USA) and in-gel trypsin digested. The digested peptide was spotted onto a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) plate in a solution containing 5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% (v/v) ACN and 0.1 % (v/v) trifluoroacetic acid. Mass spectra were recorded on a VOYAGER DE-STR mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Nd:YAG 200 Hz laser operated in positive reflector mode.

Identification of proteins from primary sequence databases was achieved by Mascot search engine, protein score identification was set > 60 and considered significant ($p<0.05$).

*Protein enrichment analysis*

Predicted protein-protein interactions and functional enrichment analysis were performed by the web resource String v.11 [41]. The minimum required interaction score was set to high confidence (0.7), the p-value for protein-protein interaction (PPI) and false discovery rate for

functional enrichment were inferior than 0.05. False discovery rate was shown as p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

### *2.5 Western Blot analysis*

Skeletal muscle specimens obtained from Group II were lysed in buffer containing 1% Triton and analyzed by Western blot as previously described [29,36-38]. 20 μg protein samples were separated using SDS gel electrophoresis (Bio-Rad Laboratories Inc.) and electrotransfered to nitrocellulose membranes (Amersham Pharmacia Biotech Inc. CA, USA).

The membranes were probed with antibodies (Abcam, Cambridge, MA, USA) against mitofilin (Catalog Number: ab110329), ATP synthase subunit alpha (ATP5A, Catalog Number: ab14748), electron transfer flavoprotein subunit alpha (ETFA, Catalog Number: ab110316), pyruvate dehydrogenase protein x component (ODPX, Catalog Number: ab110334), cytochrome C oxidase subunit VIb (CX6B1, Catalog Number: ab110266), dihydrolipoamide dehydrogenase (DLDH, Catalog Number: ab126827), dihydrolipoamide S-succinyltransferase (DLST, Catalog Number: ab187699), short chain 3-hydroxyacyl-CoA dehydrogenase (HCDH, Catalog Number: ab154088), delta(3),delta(2) enoyl-CoA isomerase (D3D2, Catalog Number: ab228544), enoyl-CoA Hydratase 1 (ECH1, Catalog Number: ab153720). Equal protein loading was confirmed by reblotting the membranes with monoclonal antibody against β-actin (Sigma-Aldrich, Milan, Italy). Blots were visualized using peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence detection, and band densities were quantified by densitometry. Skeletal muscle levels of the above indicated mitochondrial proteins were normalized to β-actin levels.

### *2.6 Statistical analysis*

Variables that were not normally distributed, including triglycerides, fasting and 2-h post-load insulin concentrations, were natural log transformed for statistical analyses. Continuous variables are expressed as means ± SD. Categorical variables were compared by $\chi 2$ test. We used a general linear model with adjustment for age, gender and BMI to test pairwise differences in mitochondrial protein levels between NGT and type 2 diabetic groups. Relationships between mitochondrial protein levels and glucose homeostasis parameters were determined by Pearson's correlation coefficient (r). Wilcoxon's signed rank test was used to test differences in clinical parameters and mitochondrial protein expression between before and after treatment with placebo or pioglitazone. P value <0.05 was considered statistically significant. All analyses were performed using SPSS software programme Version 17.0 for Windows.

## 3. Results

### 3.1 Mitochondrial proteomic analysis in Group I

A quantitative mitochondrial proteomic analysis was performed on mitochondrial enriched lysates obtained from skeletal muscle biopsies of 8 NGT and 8 type 2 diabetic subjects (Group I). The clinical characteristics of the study group I are shown in Suppl. Table 1. Figure 1 shows representative two-dimensional protein profiles of skeletal muscle mitochondrial lysates of NGT and type 2 diabetic subjects.

Mitochondrial protein profiles were highly comparable between individuals with NGT and type 2 diabetes, indicating a low level of inter-individual and experimental variation that could confound data interpretation. By performing comparative visual and software-guided analyses of these two-dimensional protein profiles coupled to mass spectrometry, among several hundred spots we identified 76 mitochondrial proteins (Suppl. Table 2) among which 12 proteins were differentially expressed in skeletal muscle of subjects with type 2 diabetes compared to NGT individuals. The list of mitochondrial proteins differentially expressed and ratio of spot volumes between type 2 diabetic and NGT groups are shown in table 1.

In particular, in skeletal muscle of type 2 diabetic subjects we found a downregulation of ATP5A, ETFA, CX6B1; all of these proteins are essential components of the mitochondrial electron transport chain. We also found a reduced abundance of ODPX, DLDH, DLST, which are enzymes involved in the Krebs cycle. Compared to NGT subjects, those with type 2 diabetes also displayed decreased skeletal muscle levels of mitofilin, a structural mitochondrial protein which plays a pivotal role in the maintenance of normal mitochondrial morphology and function [41]. Additionally, we found a higher abundance of enzymes involved in fatty acid oxidation including HCDH, D3D2 and ECH1 in skeletal muscle of subjects with type 2 diabetes compared to those with NGT.

### 3.2 Western Blot analysis in Group II

To confirm the results obtained by mitochondrial proteomic analysis, we assessed by Western blot the skeletal muscle levels of proteins involved in mitochondrial function, which were found be differentially expressed in subjects with type 2 diabetes, in a separate larger study sample encompassing 48 individuals, 24 with NGT and 24 with type 2 diabetes (Group II). Anthropometric and biochemical parameters of the subjects in Group II are shown in Table 2. Type 2 diabetic subjects were older than NGT individuals, but matched for gender, BMI, and waist circumference. As expected, type 2 diabetic subjects exhibited higher levels of fasting and 2h-post load plasma glucose, and glycated haemoglobin (HbA1c), increased plasma triglycerides, lower HDL concentrations, and reduced whole body insulin sensitivity.

After adjusting for age, gender and BMI we found that protein levels of ATP5A, ETFA and CX6B1, key factors involved in mitochondrial ATP biosynthesis, were significantly reduced by -30% ($P$=0.006), -50% ($P$=0.02) and -20% ($P$=0.03), respectively, in the skeletal muscle of subjects with type 2 diabetes compared to NGT individuals (Figure 2 A-D). Individuals affected by type 2 diabetes also displayed a decreased expression of mitofilin (-30%, $P$=0.01) (Figure 2 E) and reduced abundance of mitochondrial enzymes involved in oxidative metabolism including DLST (-20%, $P$=0.04) and ODPX (-26%, $P \leq 0.05$) as compared to NGT subjects; no significant difference in DLDH protein levels was observed (Figure 3 A-C). Additionally, we analyzed the protein expression of HCDH, ECH1 and D3D2, enzymes involved in fatty acid catabolism. After adjusting for age, gender and BMI we observed a significant increase in HCDH and ECH1 abundance (+33%, $P < 0.05$ and +29%, $P$=0.03 respectively) in the skeletal muscle of subjects with type 2 diabetes compared to NGT individuals (Figure 3 A, E-F); no significant difference between the two groups was observed for D3D2 protein levels (Figure 3 A, G). Full blot images of the above indicated mitochondrial proteins are shown in supplementary figure 1 and 2.

Additionally, we evaluated differences in skeletal muscle protein abundance of mitochondrial proteins between type 2 diabetes and NGT groups separately in men and women. We found that in women type 2 diabetes was associated with a downregulation of ATPA (-35%, *P*=0.02), ETFA (-40%, P=0.03), CX6B1 (-22%, P=0.22), DLST (-12%, P=0.11), mitofilin (-30%, P=0.05) and an upregulation of ECH1 (+27%, P=0.07). Protein levels of HCDH were not different between women with type 2 diabetes and NGT. In men, we observed that subjects with type 2 diabetes had reduced levels of ATPA (-30%, *P*=0.01), ETFA (-60%, P=0.01), CX6B1 (-33%, P=0.02), DLST (-22%, P=0.04), mitofilin (-28%, P=0.01) and an upregulation of HCDH (+50%, P=0.04) and ECH1 (+30%, P=0.04) in skeletal muscle as compared to NGT group.

Next, we evaluated the relationship between parameters of glucose homeostasis and skeletal muscle expression of mitochondrial proteins (Table 3). Univariate analysis revealed that protein levels of ATP5A, ETFA, CX6B1 and mitofilin were positively correlated with whole body insulin sensitivity and inversely with HbA1c, fasting and 2h-post load plasma glucose levels. Skeletal muscle protein levels of ODPX and DLST were positively correlated with whole body insulin sensitivity and negatively correlated with HbA1c and 2h-post load plasma glucose levels. Additionally, we found that protein abundance of ECH1 and HCDH, enzymes implicated in fatty acid oxidation, was negatively correlated with whole body insulin sensitivity and positively correlated with 2h-post load plasma glucose levels (Table 3).

### 3.3 Effect of pioglitazone treatment on skeletal muscle mitochondrial proteome

Next, we evaluated whether low-dose (15 mg/day) pioglitazone treatment could restore the content of mitochondrial proteins, which were found to be differentially expressed in skeletal muscle of subjects with type 2 diabetes compared to NGT individuals. Twenty subjects with type 2 diabetes were randomized to receive pioglitazone or placebo for 6 months. Clinical characterization

at baseline and after pioglitazone or placebo treatment is shown in Supp. Table 2. Among subjects randomized to receive placebo six were treated with metformin alone, two with metformin plus sulfonylureas and one with diet alone at baseline. In the pioglitazone group, at baseline six subjects were treated with metformin alone, three with metformin plus sulfonylureas and two with diet alone [29]. In both groups antidiabetic therapy was maintained stable during the study period and no other medications were added. The metabolic effects of pioglitazone treatment were described in a previously published study [29]. Briefly, diabetic subjects treated with pioglitazone for 6 months displayed lower levels of fasting and 2h-post load glucose, HbA1c, triglycerides, total and low density lipoprotein (LDL) cholesterol, and improved whole body insulin sensitivity as compared to baseline (Suppl. Table 3).

Notably, after 6 months of treatment with low-dose pioglitazone skeletal muscle protein levels of ATP5A, ETFA and CX6B1 were significantly increased by +33% ($P \leq 0.05$), +60% ($P \leq 0.05$) and +33% ($P=0.01$), respectively, compared to baseline, whereas no change was observed in patients randomized to placebo (Figure 4 A-D). Subjects treated with pioglitazone, but not those randomized to placebo, exhibited a 20% increase ($P \leq 0.05$) in the skeletal muscle abundance of mitofilin compared to baseline (Figure 4 E). We observed an increase in skeletal muscle DLST levels in the pioglitazone-treated group compared to baseline, although it did not reach the threshold for statistical significance (+10%, p=0.08) (Figure 5 A-B). Protein levels of ODPX were not altered by either pioglitazone or placebo treatment (Figure 5 A-C).

HCDH and ECH1 protein levels, which were upregulated in skeletal muscle of subjects with type 2 diabetes, were reduced by -10% and -15%, respectively ($P \leq 0.05$ for both) after pioglitazone treatment (Figure 5 D-E).

### 3.4 Functional protein-protein interaction analysis

Figure 6 shows the functional protein-protein interaction taking into consideration both the

biochemical abnormalities in type 2 diabetes and the effect of low-dose pioglitazone treatment on skeletal muscle mitochondrial proteome. Subjects with type 2 diabetes exhibit reduced skeletal muscle levels of mitochondrial proteins involved in oxidative phosphorylation, oxidation-reduction processes, production of ATP and in the Krebs cycle (downregulation of CX6B1, ATP5A, ETFA, ODPX, mitofillin, DLDH and DLST). On the contrary, type 2 diabetes was characterized by the overexpression of proteins involved in metabolic process and especially on fatty acids beta-oxidation (upregulation of ECH1 and HCDH). Notably, the administration for 6 months of pioglitazone is able to exert a positive effect on type 2 diabetes related abnormalities in mitochondrial proteome, especially restoring oxidative metabolism (upregulation of ATP5A, ETFA, mitofillin and CX6B1) and reducing protein levels of enzymes implicated in fatty acids catabolism (downregulation of HCDH and ECH1).

## 4. Discussion

Several mitochondria abnormalities have been described in skeletal muscle of subjects with type 2 diabetes [4-10].

However, the results of these studies were based on oligonucleotide microarray approaches or proteomic analysis by two-dimensional differential-gel electrophoresis performed on a small number of individuals [22-28] and did not examine whether these abnormalities could be improved by an intervention known to ameliorate skeletal muscle insulin resistance such as pioglitazone [29,31]. In order to identify abnormalities associated with mitochondrial dysfunction in skeletal muscle of type 2 diabetic subjects, we first compared the skeletal muscle mitochondrial proteomic profile in diabetic subjects with that of NGT individuals using 2D-gel electrophoresis coupled with mass spectrometry-based protein identification. Next, we confirmed our observations by performing Western blot analysis on skeletal muscle lysates obtained in a separate and larger study group.

Our results demonstrate that skeletal muscle abundance of mitochondrial enzymes involved in ATP production and oxidative metabolism is reduced in type 2 diabetic individuals as compared to those with NGT, even after adjustment for BMI, age and gender. Protein levels of key components of the mitochondrial electron transport chain (ATP5A, ETFA and CX6B1) and mitochondrial enzymes involved in Krebs cycle (DLST and ODPX) were significantly reduced in the skeletal muscle of subjects with type 2 diabetes. Levels of mitofilin, a protein anchored to the mitochondrial inner membrane, which has been shown to play a critical role in maintaining the mitochondrial structural and functional integrity [42], was also decreased in diabetic subjects. These results are in agreement with previous reports suggesting an association between type 2 diabetes and reduced electron transport chain and Krebs cycle activity and altered mitochondrial structural integrity in skeletal muscle [7-9,43]. Despite the gender releted differences in pathogenic mechanisms of type 2 diabetes [44-46], we observed that the directional changes, as well as the magnitude of the changes in mitochondrial protein levels were similar in males and females.

The reduced abundance of mitochondrial proteins involved in oxidative phosphorylation in type 2 diabetic subjects was associated with an increased expression of HCDH and ECH1, enzymes implicated in fatty acid oxidation. With regard to this, conflicting results have been published with studies demonstrating both decreased and increased fatty acid oxidative enzyme expression and/or activity in type 2 diabetic individuals [4,24,43,47,48]. Some studies have suggested that skeletal muscle fatty acid oxidation is reduced in type 2 diabetes and that the impairment in fat oxidation contributes to insulin resistance by promoting the accumulation of toxic intracellular lipid metabolites [24,48, 49]. However, increasing evidence has suggested that both obesity and type 2 diabetes are associated with increased lipid oxidation, which is incomplete and not coupled to a higher rate of mitochondrial respiration [5,43,50]. Consistent with this proposed model, we found that individuals with type 2 diabetes exhibited increased skeletal muscle levels of enzymes involved in fatty acid catabolism, accompanied by reduced abundance of proteins involved in downstream

metabolic pathways, such as the Krebs cycle and electron transport chain. The imbalance between β-oxidation and Krebs cycle/electron transport chain activity may promote intracellular accumulation of incompletely metabolized lipids [15,16], resulting in an elevated concentration of intra-mitochondrial and cytoplasmic NADH [8]. A higher NADH/NAD ratio is known to impair pyruvate dehydrogenase, driving pyruvate to oxaloacetate and citrate [11,51]. Impaired citrate consumption in the Krebs cycle could lead to its accumulation in mitochondria and cytoplasm, where it is used as a precursor of acetyl-CoA, malonyl-CoA, and diacyl- and triacylglycerols [8,52]. Further, increased levels of NADH can inhibit the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and, together with the accumulation of pyruvate, lead to accumulation of glycolytic intermediates. This would increase flux into the polyol, hexosamine, protein kinase C and advanced glycation end-product pathways, thereby worsening insulin resistance and glucose metabolism [11]. Consistent with this scenario, we found a close correlation between the abundance of several mitochondrial proteins involved in the Krebs cycle and electron chain transport versus insulin sensitivity and glucose control, including HbA1c and 2-hour plasma glucose during OGTT. To the contrary, levels of enzymes involved in fatty acid oxidation, ECH1 and HCDH, were negatively associated with insulin sensitivity and positively correlated with 2-hour plasma glucose levels.

In the present study we also evaluated the *in vivo* effect of low-dose pioglitazone on mitochondrial proteome profile. We found that the improvement in insulin sensitivity and glucose homeostasis in type 2 diabetic subjects treated with low-dose pioglitazone for 6 months was associated with a restored expression of the electron transport chain components ATP5A, CX6B1 and ETFA and a significant increase in the abundance of mitofilin. Moreover, the up-regulated skeletal muscle expression of ECH1 and HCDH in subjects with diabetes was decreased by pioglitazone treatment, indicating that pioglitazone improved the diabetes-related aberrant abundance of mitochondrial proteins. These results support previous evidence suggesting that

pioglitazone improves skeletal muscle mitochondrial health by increasing the transcript levels of genes involved in oxidative metabolism and reducing muscle β-oxidation capacity [30,32,53]. However, there are reports in which no effect of pioglitazone on muscle mitochondrial activity and its influence on in-vivo glucose metabolism was observed [54-55]. The difference between these previous results could be explained by differences in the patient population, i.e. nondiabetic subjects with the metabolic syndrome were studied by Yokata et al [54], and differences in the study duration and dose of pioglitazone [54-55]. Further, in this previous study no increase in insulin-stimulated whole body glucose disposal was observed [54]. Therefore, one would not expect any change in mitochondrial function.

The present study has some strengths including the analysis of skeletal muscle mitochondrial proteome performed by 2D-gel electrophoresis and tandem-mass-spectrometry, the validation of the results obtained by 2D-gel based proteomic approach by Western blot analysis performed on a larger sample, detailed anthropometric and biochemical characterization including OGTT and clamp procedures to evaluate glucose homeostasis and whole body insulin sensitivity, assessment of mitochondrial protein abundance in skeletal muscle of type 2 diabetic individuals before and after treatment with pioglitazone or placebo.

However, there are some limitations of the present study. First, there was a difference in age between the diabetic and control groups. Although aging may affect expression and activity of mitochondrial enzymes [56], the observed differences in mitochondrial protein levels between the diabetic and the control groups were not affected by adjustment for age. Another limitation of this proteomic analysis is that we did not evaluate post-translational modifications of proteins as previously reported by others [26,27]. Aruda et al. have demonstrated differences in mitochondria-associated ER membranes in obese individuals, raising the possibility that structural mitochondrial alterations are present in obesity/T2DM [57]. Thus, a limitation of this study is that we did not examine whether structural alteration of mitochondrial proteins could account for some of the

differences that we observed between T2DM and NGT subjects. Second, mitochondrial enzymatic activities were not assessed in the skeletal muscle biopsies. Thus, it remains to be determined whether the altered abundance of proteins involved in oxidative metabolism found in diabetic subjects is translated to altered enzymatic activity of these proteins. Third, although there is a close relationship between altered levels of multiple skeletal muscle proteins and both insulin resistance and impaired glucose homeostasis, these do not prove causality.

## 5. Conclusion

In conclusion, our findings demonstrate that subjects with type 2 diabetes have reduced levels of mitochondrial proteins involved in oxidative phosphorylation and an increased abundance of enzymes involved in fatty acid catabolism in the skeletal muscle. Importantly, pioglitazone at low-dose is able to exert a positive effect on the abnormal mitochondrial protein profile in the skeletal muscle of subjects with type 2 diabetes.

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Authors contribution: TVF wrote and edited the manuscript, performed WB experiments and analyzed the data. AM, GD, AOC, MAG collected the data. AM and SK performed proteomic analysis. SK and RS contributed to WB analyses. MDC performed protein-protein interaction and functional analysis. DT, MAG, MLH, GS contributed to the discussion and to the interpretation of the data. RAD reviewed and corrected the manuscript. FF conceived the study, interpreted data and reviewed the manuscript. FF is the guarantor of this work and, as 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.

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**Figures legend**

**Figure 1.** Representative images of two-dimensional protein profiles of skeletal muscle mitochondrial lysates of subjects with normal glucose tolerance (NGT) and type 2 diabetes mellitus (DM).

ATP5A: ATP synthase alpha chain; ETFA: electron transfer flavoprotein alpha-subunit; CX6B1: cytochrome c oxidase subunit VIb isoform 1; ODPX: pyruvate dehydrogenase protein X component; DLDH: dihydrolipoamide dehydrogenase; DLST: dihydrolipoamide S-succinyltransferase; HCDH: hydroxyacyl-coenzyme A dehydrogenase; D3D2: 3,2-trans-enoyl-CoA isomerase; ECH1: delta3,5-delta2,4-dienoyl-CoA isomerase; AK3: adenylate Kinase 3; CA3: carbonic anhydrase 3.

**Figure 2.** Representative western blot images (A) and quantification of skeletal muscle ATP5A (B), ETFA (C), CX6B1 (D), and mitofilin (E) in subjects with type 2 diabetes (DM) and normal glucose tolerance (NGT).

Band densities were quantified by densitometry. Skeletal muscle levels of the mitochondrial proteins were normalized to β-actin levels. P values reported above every graph refers to analyses adjusted for age, gender and BMI. NOD: normalized optical density.

**Figure 3.** Representative western blot images (A) and quantification of skeletal muscle DLST (B), ODPX (C), DLDH (D), HCDH (E), ECH1 (F), D3D2 (G) in subjects with type 2 diabetes (DM) and normal glucose tolerance (NGT).

Band densities were quantified by densitometry. Skeletal muscle levels of the mitochondrial proteins were normalized to β-actin levels. P values reported above every graph refers to analyses adjusted for age, gender and BMI. NOD: normalized optical density.

**Figure 4.** Representative western blot images (A) and quantification of skeletal muscle ATP5A, ETFA, CX6B1 and mitofilin at baseline (BAS) and after treatment with placebo (PLC) or pioglitazone (PIO) for 6 months (end of the study, EOS) in subjects with type 2 diabetes. Band densities were quantified by densitometry. Skeletal muscle levels of the mitochondrial proteins were normalized to β-actin levels. Comparison between baseline and after treatment data were performed by Wilcoxon's signed rank test. NOD: normalized optical density.

**Figure 5.** Representative western blot images (A) and quantification of skeletal muscle DLST, ODPX, HCDH and ECH1 at baseline (BAS) and after treatment with placebo (PLC) or pioglitazone (PIO) for 6 months (end of the study, EOS) in subjects with type 2 diabetes. Band densities were quantified by densitometry. Skeletal muscle levels of the mitochondrial proteins were normalized to β-actin levels. Comparison between baseline and after treatment data were performed by Wilcoxon's signed rank test. NOD: normalized optical density.

**Figure 6.** Functional protein-protein interaction in (**A**) healthy and (**B**) type 2 diabetes skeletal muscle mitochondrial proteome. The protein network is divided into clusters (color-coded) accordingly to the protein biological process. In the figure are evidenced oxidation-reduction process (gene onthology, GO:0055114, green), fatty acid beta-oxidation (GO:0006635, lime), generation of precursor metabolites and energy (GO:0006091, pink), small molecule metabolic process (GO:0044281, red), and carboxylic acid catabolic process (GO:0019752, blue). The arrows indicate the modulation of the protein expression in type 2 diabetes (upregulated in teal and downregulated in light teal) and in type 2 diabetes after six months of low-dose pioglitazone (upregulated in orange and downregulated in light orange). The thickness of the grey lines between edges indicates the strength of data support. The minimum required interaction score was set to high confidence (>0.7), false discovery rate, and PPI enrichment p-value were in here inferior to 0.05.

**Table 1. List of 2D gel electrophoresis-identified proteins differentially expressed in skeletal muscle of subjects with type 2 diabetes in comparison to NGT individuals**

| | DM/NGT ratio | *P* |
|---|---|---|
| ATP synthase alpha chain (ATP5A) | 0.46 | 0.03 |
| Electron transfer flavoprotein alpha-subunit (ETFA) | 0.78 | 0.04 |
| Cytochrome c oxidase subunit VIb isoform 1 (CX6B1) | 0.71 | 0.03 |
| Pyruvate dehydrogenase protein X component (ODPX) | 0.55 | 0.02 |
| Dihydrolipoamide dehydrogenase (DLDH) | 0.80 | 0.03 |
| Dihydrolipoamide S-succinyltransferase (DLST) | 0.54 | 0.05 |
| Mitofilin | 0.59 | 0.02 |
| Hydroxyacyl-coenzyme A dehydrogenase (HCDH) | 1.33 | 0.05 |
| 3,2-trans-enoyl-CoA isomerase (D3D2) | 1.27 | 0.05 |
| Delta3,5-delta2,4-dienoyl-CoA isomerase (ECH1) | 1.35 | 0.03 |
| Adenylate Kinase 3 (AK3) | 1.42 | 0.02 |
| Carbonic anhydrase 3 (CA3) | 1.81 | 0.02 |

List of spot proteins with different expression in subjects with type 2 diabetes mellitus (DM) in comparison to those with normal glucose tolerance (NGT). Ratio of spot volumes between DM and NGT is shown.

**Table 2. Clinical characterization of individuals of study group II stratified according to glucose tolerance**

| | NGT | DM | *P* |
|---|---|---|---|
| n *(M/F)* | 12/12 | 14/10 | 0.56 |
| Age *(yrs)* | 39±12 | 57±7 | ≤0.001* |
| Anti-diabetic therapy | | Diet 5<br>Met 13<br>Met+Sulf 6 | |
| BMI *(kg/m$^2$)* | 30±7 | 32±6 | 0.29 |
| Waist circumference *(cm)* | 97.4±13 | 96.2±17 | 0.98 |
| Systolic blood pressure *(mmHg)* | 125±14 | 131±12 | 0.25 |
| Diastolic blood pressure *(mmHg)* | 73±11 | 73±9 | 0.67 |
| Fasting glucose *(mg/dl)* | 94±5 | 150±37 | ≤0.001 |
| 2-h glucose (*mg/dl)* | 118±27 | 277±51 | ≤0.001 |
| HbA1c (*%)* | 5.3±0.3 | 7.5±1.2 | ≤0.001 |
| M-value (*mg/kg·min*) | 7.7±2.4 | 3.5±1.5 | ≤0.001 |
| Fasting insulin *(mUI/ml)* | 4.06±3.2 | 11.1±11 | 0.13 |
| 2-h insulin *(mUI/ml)* | 37±19 | 40±29 | 0.77 |
| Total cholesterol (*mg/dl* | 174±27 | 172±36 | 0.42 |
| HDL cholesterol (*mg/dl)* | 46±11 | 39±8 | 0.02 |
| LDL cholesterol (*mg/dl)* | 104±25 | 95±33 | 0.37 |
| Triglycerides (*mg/dl)* | 103±58 | 120±96 | 0.03 |

Data are means ± SD. Triglycerides, fasting and 2-h post-load insulin were log transformed for statistical analysis, but values in the table represent back transformation to the original scale. Categorical variables were compared by $\chi 2$ test. Comparisons between groups were performed using a general linear model. P values refer to results after analyses with adjustment for age, and gender. *P values refer to results after analyses with adjustment for gender. BMI: body mass index; HbA1c: glycated hemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein.

**Table 3- Univariate correlations between mitochondrial protein levels in skeletal muscle and glucose homeostasis parameters**

| | Whole body insulin sensitivity | | Fasting plasma glucose | | 2h-post load plasma glucose | | HbA1c | |
|---|---|---|---|---|---|---|---|---|
| | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* |
| ATP5A | 0.60 | ≤0.0001 | -0.44 | 0.002 | -0.49 | ≤0.0001 | -0.51 | ≤0.0001 |
| ETFA | 0.37 | 0.005 | -0.40 | 0.003 | -0.55 | ≤0.0001 | -0.51 | ≤0.0001 |
| CX6B1 | 0.41 | 0.007 | -0.37 | 0.01 | -0.33 | 0.03 | -0.45 | 0.003 |
| Mitofilin | 0.34 | 0.02 | -0.39 | 0.005 | -0.37 | 0.01 | -0.35 | 0.02 |
| DLST | 0.31 | 0.03 | -0.21 | 0.15 | -0.36 | 0.01 | -0.29 | 0.05 |
| ODPX | 0.28 | 0.04 | -0.16 | 0.23 | -0.32 | 0.02 | -0.35 | 0.01 |
| DLDH | 0.06 | 0.65 | -0.05 | 0.69 | -0.04 | 0.75 | -0.07 | 0.61 |
| HCDH | -0.27 | 0.05 | 0.19 | 0.16 | 0.29 | 0.03 | 0.26 | 0.09 |
| ECH1 | -0.38 | 0.03 | 0.27 | 0.13 | 0.32 | 0.05 | 0.27 | 0.15 |
| D3D2 | 0.18 | 0.29 | -0.28 | 0.09 | -0.33 | 0.06 | -0.34 | 0.06 |

**CRediT author statement**

Teresa Vanessa Fiorentino: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Review & Editing

Adriana Monroy and Subash Kamath: Writing - Original Draft, Investigation, Resources

Giuseppe Daniele, Muhammad Abdul-Ghani: Investigation and Data Curation

Rosa Sotero: Investigation

Michele Dei Cas: Formal analysis, Writing - Review & Editing

Alberto O. Chavez: Investigation

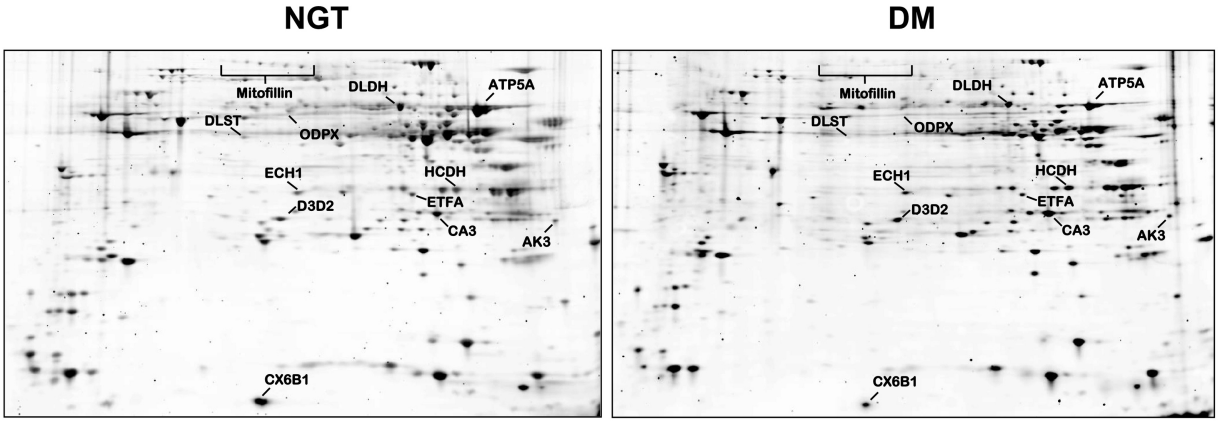
Marta Letizia Hribal, Giorgio Sesti, and Devjit Tripathy: Visualization, Writing - Review & Editing

Ralph A. DeFronzo: Visualization, Supervision, Project administration, Writing - Review & Editing, Funding acquisition

Franco Folli: Conceptualization, Visualization, Supervision, Project administration, Writing - Review & Editing, Funding acquisition

**Highlights**

- Mitochondrial proteomic analysis on human skeletal muscle biopsies reveals that type 2 diabetes is associated with an altered mitochondrial proteomic profile.
- Subjects with type 2 diabetes exhibit a reduced skeletal muscle abundance of key components of the mitochondrial electron transport chain and enzymes involved in Krebs cycle.
- Type 2 diabetes is associated with an upregulation of enzymes implicated in fatty acid oxidation.
- Pioglitazone treatment reverts the diabetes-related abnormalities in the skeletal muscle expression of mitochondrial proteins involved in oxidative metabolism.


NGT
Mitofillin
DLDH
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Mitofillin
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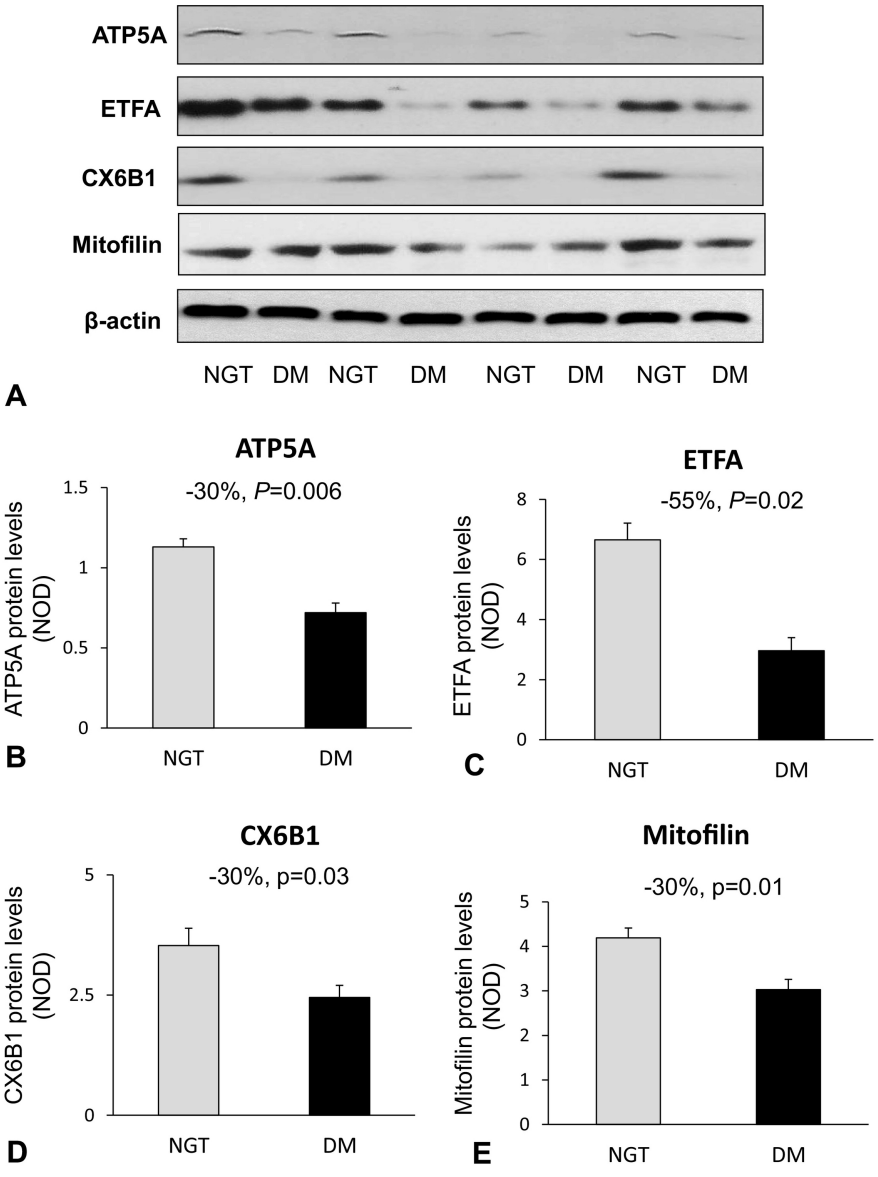
AK3
CX6B1

Figure 1


ATP5A
ETFA
CX6B1
Mitofilin
β-actin
NGT DM NGT DM NGT DM NGT DM
A
ATP5A
-30%, P=0.006
ATP5A protein levels (NOD)
1.5
1
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B
ETFA
-55%, P=0.02
ETFA protein levels (NOD)
8
6
4
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C
CX6B1
-30%, p=0.03
CX6B1 protein levels (NOD)
5
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Mitofilin
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Mitofilin protein levels (NOD)
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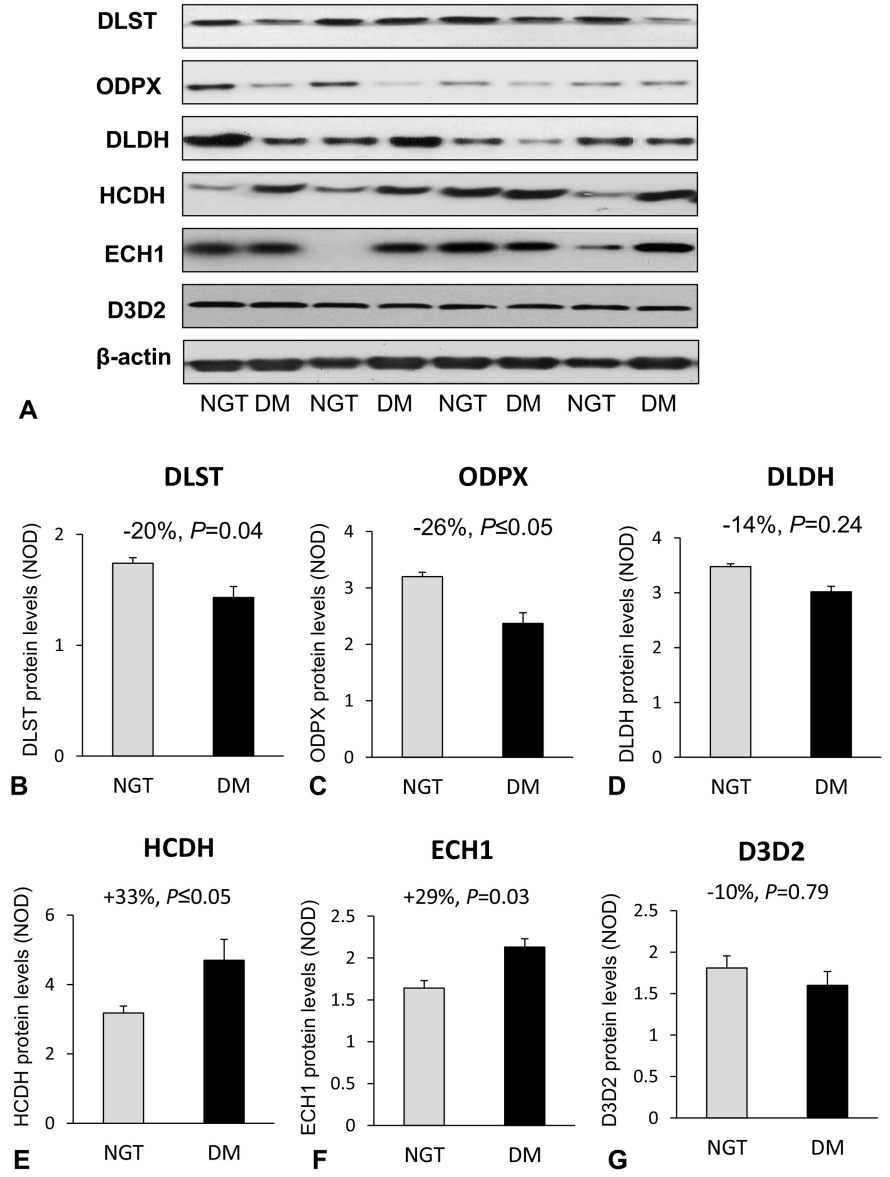
Figure 2


DLST
ODPX
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NGT DM NGT DM NGT DM NGT DM
A
DLST
-20%, P=0.04
DLST protein levels (NOD)
B
NGT
DM
ODPX
-26%, P≤0.05
ODPX protein levels (NOD)
C
DLDH
-14%, P=0.24
DLDH protein levels (NOD)
D
HCDH
+33%, P≤0.05
HCDH protein levels (NOD)
E
ECH1
+29%, P=0.03
ECH1 protein levels (NOD)
F
D3D2
-10%, P=0.79
D3D2 protein levels (NOD)
G

Figure 3

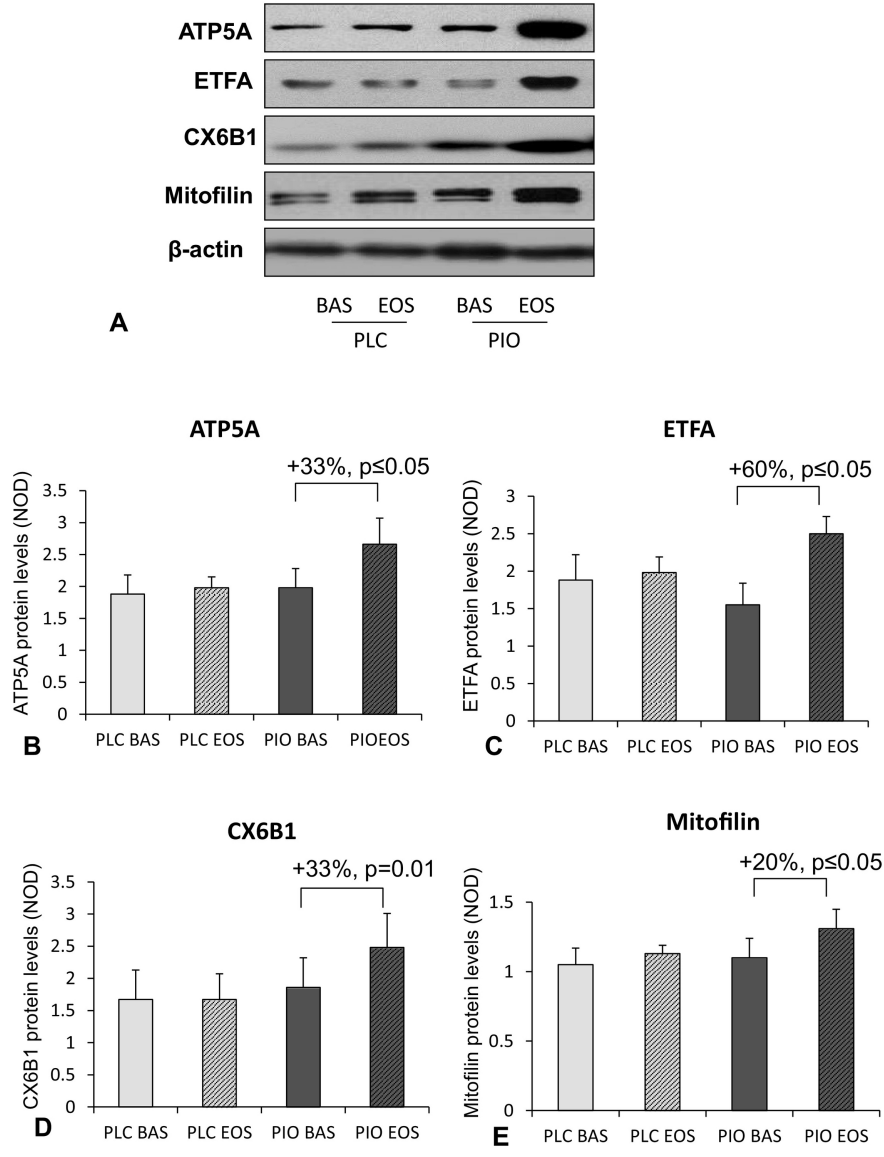
ATP5A
ETFA
CX6B1
Mitofilin
β-actin
BAS EOS BAS EOS
PLC PIO
A
ATP5A
+33%, p≤0.05
ATP5A protein levels (NOD)
PLC BAS PLC EOS PIO BAS PIOEOS
B
ETFA
+60%, p≤0.05
ETFA protein levels (NOD)
PLC BAS PLC EOS PIO BAS PIO EOS
C
CX6B1
+33%, p=0.01
CX6B1 protein levels (NOD)
PLC BAS PLC EOS PIO BAS PIO EOS
D
Mitofilin
+20%, p≤0.05
Mitofilin protein levels (NOD)
PLC BAS PLC EOS PIO BAS PIO EOS
E

Figure 4

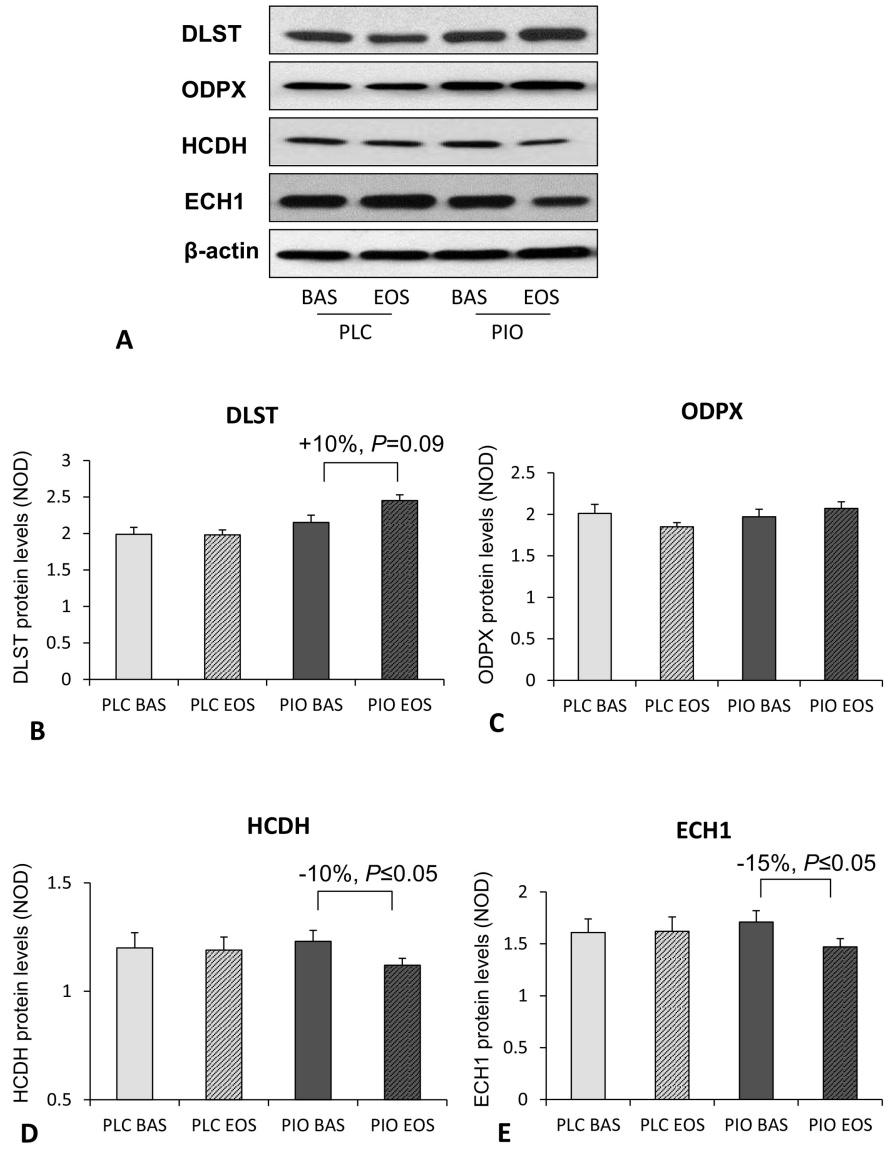
DLST
ODPX
HCDH
ECH1
β-actin
BAS
EOS
BAS
EOS
PLC
PIO
A
DLST
+10%, P=0.09
DLST protein levels (NOD)
PLC BAS
PLC EOS
PIO BAS
PIO EOS
B
ODPX
ODPX protein levels (NOD)
C
HCDH
-10%, P≤0.05
HCDH protein levels (NOD)
D
ECH1
-15%, P≤0.05
ECH1 protein levels (NOD)
E

Figure 5

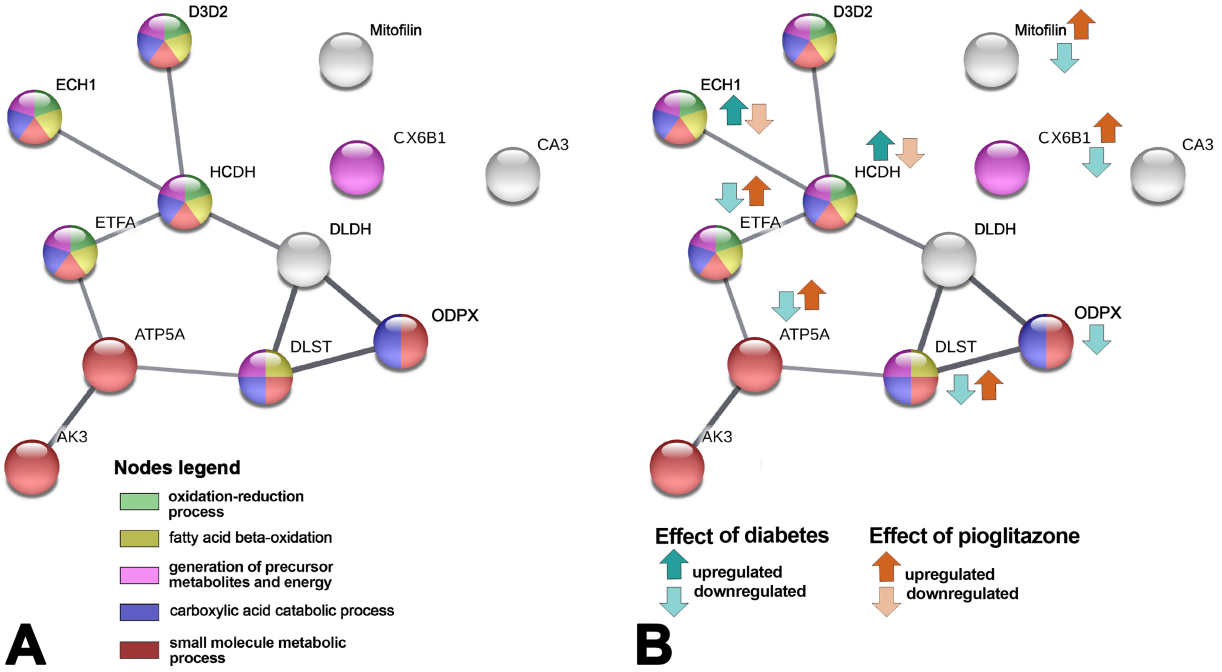
D3D2
Mitofilin
ECH1
CX6B1
CA3
HCDH
ETFA
DLDH
ODPX
ATP5A
DLST
AK3
Nodes legend
oxidation-reduction process
fatty acid beta-oxidation
generation of precursor metabolites and energy
carboxylic acid catabolic process
small molecule metabolic process
A
B
Effect of diabetes
upregulated
downregulated
Effect of pioglitazone
upregulated
downregulated

Figure 6