TGFβ contributes to impaired exercise response by suppression of mitochondrial key regulators in skeletal muscle

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Abstract:

A substantial number of people at risk to develop type 2 diabetes could not improve insulin sensitivity by physical training intervention. We studied the mechanisms of this impaired exercise response in 20 middle-aged individuals at high risk to develop type 2 diabetes who performed a controlled eight weeks cycling and walking training at 80 % individual VO_2peak. Participants identified as non-responders in insulin sensitivity (based on Matsuda index) did not differ in pre-intervention parameters compared to high responders. The failure to increase insulin sensitivity after training correlates with impaired up-regulation of mitochondrial fuel oxidation genes in skeletal muscle, and with the suppression of the upstream regulators PGC1α and AMPKα2. The muscle transcriptome of the non-responders is further characterized by an activation of TGFβ and TGFβ target genes, which is associated with increases in inflammatory and macrophage markers. TGFβ1 as inhibitor of mitochondrial regulators and insulin signaling is validated in human skeletal muscle cells. Activated TGFβ1 signaling down-regulates the abundance of PGC1α, AMPKα2, mitochondrial transcription factor TFAM, and of mitochondrial enzymes. Thus, the data suggest that increased TGFβ activity in skeletal muscle can attenuate the improvement of mitochondrial fuel oxidation after training and contribute to the failure to increase insulin sensitivity.

Introduction

Type 2 diabetes has become an epidemic disease with more than 350 million affected people worldwide. The importance of physical activity to prevent or ameliorate the diabetic metabolic state and its consequences is well-accepted and regular exercise is recommended both in prevention and treatment of type 2 diabetes (1-3). Regularly performed, it increases whole body fat oxidation and insulin sensitivity (4-6). However, evidence is accumulating in the last years that differences exist in the individual response to exercise training. On average, approximately 15 – 20% of people failed to improve their glucose tolerance and insulin sensitivity after exercise training interventions (7) and this non-response is not restricted to a specific type of exercise (endurance or resistance training or combinations) albeit differences are reported (8-11).

The molecular basis for this individual response to exercise intervention is unclear. Genome-wide linkage scans performed in the HERITAGE family study underline the importance of genetic factors for the individual response of metabolic parameters to exercise (12;13). We hypothesize that the individual response is related to specific transcriptional signatures after training intervention that elucidate the relevant changes in muscle gene expression pattern that are linked to improvement of insulin sensitivity. Moreover, whole genome expression signatures can indicate the molecular mechanisms responsible for the lack of improvement in metabolic parameters after exercise training. To this end, we studied the training intervention outcome of twenty individuals at high risk for type 2 diabetes. We assessed insulin sensitivity using the surrogate Matsuda insulin sensitivity index (ISI) (14), anthropometric and clinical parameters, and physical fitness before and after an eight-weeks supervised endurance training. Responders and non-responders in insulin sensitivity were defined based on the fold change in insulin sensitivity before and after intervention. Whole genome microarray analysis of skeletal muscle

biopsies obtained before and after training was performed. These data suggested that enhanced TGFβ1 signaling can be a negative regulator of exercise response and this role was further investigated in primary human skeletal muscle cells.

Research Design and Methods

Study participants and phenotyping

20 middle-aged (mean 46.4±11.0 years, table S1) and sedentary (less than 2h habitually physical activity/week; mean VO2peak (bike) 22.9±5.1 ml/min/kg, table S1) individuals at high risk for type 2 diabetes were recruited. They met at least one of the following inclusion criteria: BMI >27 kg/m^2, family history (first grade) of type 2 diabetes, or former gestational diabetes. By routine laboratory tests and physical examination, severe diseases were excluded. Pre and post intervention, insulin sensitivity (ISI) was determined by a 75 g oral glucose tolerance test and calculated by the method of Matsuda and DeFronzo (14). Clinical chemical routine parameters were measured with the ADVIA 1650 clinical chemical analyzer; insulin was analyzed with the ADVIA Centaur immunoassay system (both Siemens Healthcare Diagnostics, Fernwald, Germany). Body fat mass and distribution were determined by magnetic resonance imaging (15). Informed written consent was given by all individuals; the study protocol has been approved by the ethics committee of the University of Tübingen and was in accordance with the declaration of Helsinki.

Exercise test and training intervention

Before and after the training period, all participants underwent lactate diagnostics and maximal spiroergometry in both, an incremental cycling test using an electromagnetically braked bicycle ergometer (Excalibur Sport, Lode BV, Groningen, Netherlands) and an incremental walking test on a motor-driven treadmill ergometer (Saturn 2.0 and Pulsar 4.0; HP-Cosmos, Traunstein, Germany) to determine individual lactate threshold (IAT) and VO_2peak (table 1). The test was terminated at volitional exhaustion or muscular fatigue. IAT was defined according to (16). Peak

VO_2 was defined as the mean VO_2 over the last 20 seconds prior to cessation of exercise and assessed by metabolic gas analysis (MetaLyzer 3B and MetaMax 3B, Cortex Biophysics GmbH, Leipzig, Germany).

The training program lasted 8 weeks and consisted of three supervised exercise sessions per week. Each training session consisted of 30 min each bicycle ergometer exercise and 30 min walking on a treadmill. The training intensity was individually set at 80% of the VO_2peak determined in the performance test before intervention and not changed throughout the training period. Training intensity was controlled by heart rate and estimated as energy expenditure (kJ/kg/min) according to (17).

Muscle biopsies and microarray analysis

Muscle biopsies were taken from the lateral portion of the vastus lateralis of the quadriceps femoris after local anesthesia (2% scandicaine, Astra Zeneca, Wedel, Germany) before and after the training period, under sterile conditions using a fine needle punch biopsy technique (Peter Pflugbeil GmBH, Zorneding, Germany). Snap-frozen human muscle biopsies were homogenised using a TissueLyser II (Qiagen, Hilden, Germany). Total RNA was isolated employing the miRNeasy Mini kit (Qiagen) including DNase digestion. The Agilent 2100 Bioanalyzer was used to assess RNA quality and only high quality RNA (RIN>7) was used for microarray analysis. Total RNA (30 ng) was amplified using the Ovation Pico WTA System V2 in combination with the Encore Biotin Module (Nugen). Amplified cDNA was hybridised on Affymetrix Human Transcriptome Array 2.0 (Santa Clara, CA, USA). Staining and scanning were done according to the Affymetrix protocol including minor modifications as suggested in the Encore Biotin protocol. RNA was not available of one subject of the NRE group. One sample (HRE group) was

excluded from the analysis due to low quality data. Array data has been submitted to GEO (GSE72462).

Material and Reagents

Recombinant TGFβ1 protein, and TGFβ1 and PF4 ELISA were from R&D systems (Minneapolis, MN, USA), TGFBI ELISA from Abcam (Cambridge, UK). Cell culture media and supplements were from Lonza (Basel, Switzerland), chicken embryo extract was from Seralab (West Sussex, UK). Antibodies against ATP5A (ab110273) and GAPDH (ab8245) were from Abcam (Cambridge, UK), against AMPKα2 (07-363) from Upstate Biotechnology (Lake Placid, NY, USA), against phospho-Ser-473 and phospho-Thr-308 of Akt (9271 and 9275) and RPS6 (2217) from Cell Signaling Technology (Frankfurt, Germany), and against Akt (610861) from BD Biosciences (Heidelberg, Germany).

Human skeletal muscle cell experiments

Skeletal muscle cells were obtained from six randomly selected donors including four participants of the present study. Cells were grown from satellite cells obtained from percutaneous needle biopsies performed on the lateral portion of quadriceps femoris (vastus lateralis) muscle. The donors gave informed written consent to the study. The Ethical Committee of the Tübingen University Medical Department had approved the protocol. CD56-positive myoblasts were isolated using MACS microbeads (Milteny Biotech, Bergisch Gladbach, Germany) and grown on 6-well plates coated with Geltrex (Life Technologies, Frankfurt, Germany) in a 1:1 mixture of α-MEM and Ham's F-12 supplemented with 20 % FBS, 1 % chicken extract, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml amphotericin B until

70-80 % confluency. TGFβ1 treatment was started at day 1 (for 7 days), or day 4 (for 3 days) of fusion; cells were fused in α-MEM containing 5.5 mM glucose with 2 % FBS, 2 mM glutamine, 125 µM palmitate, 125 µM oleate, 100 µM L-carnitine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml amphotericin B. RNA and proteins were isolated using the NucleoSpin miRNA Kit (Macherey Nagel, Düren, Germany).

Quantitative PCR analysis

RNA was transcribed by reverse transcription PCR with random hexamer and oligo-dT primers mixed using the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). Quantitative real time PCR (qPCR) was performed on a Roche Lightcycler 480 using QuantiTect Primer Assays (Qiagen, Hilden, Germany, table 2),

Western blotting

Protein concentration was quantified with the protein quantification assay (Macherey Nagel, Düren, Germany). Proteins were separated by sodium dodecyl sulfate polyacrylamide (7.5-15%) gradient gel electrophoresis and were transferred onto a nitrocellulose membrane by semi-dry electroblotting. Immunodetection was performed as recently described (18).

Immunostaining

Cells grown on cover slips were washed with PBS, fixed in PBS containing 4% formaldehyde (pH 7.4) for 20 min, quenched with 150 mM glycine in PBS for 10 min and treated with 0.1 % Triton X-100 for 2 min. Blocking was performed in 1% NGS, 0.05% Tween 20 in PBS for 30 min. Cover slips were incubated with antibodies recognizing CD56 (5.1H11; DSHB Iowa City,

IA, USA), MHC-fast (M4276), or MHC-slow (M8421; Sigma-Aldrich, Deisenhofen, Germany) diluted 1:100 in blocking solution for 1 h at room temperature, washed three times in PBS and incubated with the Alexa 488-labelled secondary antibody (Invitrogen, Karlsruhe, Germany) diluted 1:250 in blocking solution for another 2 h and washed again. Nuclei were stained using TO-PRO3 (Invitrogen, Karlsruhe, Germany) before mounting in PermaFluor (Beckman Coulter, Krefeld, Germany).

ELISA

TGFβ1 and TGFβ-inducible protein (TGFBI) were determined in plasma samples obtained after an overnight fast by ELISA. To correct for acute platelet activation during sample preparation platelet factor 4 (PF4/CXCL4) was measured in parallel (19).

Statistical analysis

For clinical data, statistical analysis was done using JMP11 (SAS Institute, USA). If not stated otherwise, a two-sided homoscedastic t-test was performed. For time*group interaction, mANOVA was performed. A p-value <0.05 was considered statistically significant. For some variables, participants had to be excluded due to incomplete data sets (pre/post intervention); if so, number of analyzed participants was stated. If outliers were detected (using Grubbs' test (maximum normed residual test) via http://graphpad.com/quickcalcs/grubbs1/), number of individuals included was stated. Differential gene regulation between HRE and NRE samples was done by applying the limma t-test on log2 ratios (PI vs BL; p-value < 0.05). To reduce background signals data was filtered for linear arbitrary expression >8 in at least half of the samples. The microarray data set was analyzed by the statistical programming environment R

implemented in CARMAweb (20) developed for microarray data analysis. Genewise testing for differential abundance at baseline or post intervention between ISI high- and non-responders was done by the paired limma t-test and Benjamini-Hochberg multiple testing correction (FDR < 10%, average abundance in at least one group > 8). Enriched KEGG pathways and GO terms were identified using InCroMAP (21). Upstream regulator analysis was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Results

Individual response to exercise training

After eight weeks of supervised training the entire middle-aged study group achieved an increase in the individual anaerobic threshold (IAT) and oxygen uptake (VO_2peak (bike)), a decrease in BMI, total adipose tissue, blood pressure, resting heart rate, plasma triglycerides and LDL cholesterol (table S1). Insulin sensitivity (ISI) did not increase significantly in the entire study group (8.3±6.4 vs. 9.5±7.8; p=0.1), with a high variability in the change of ISI, ranging from 2.7-fold to 0.6-fold post-intervention. Two groups with the largest difference in the fold change of ISI (8 high-responder (HRE) and 8 non-responder (NRE) were stratified by the two outermost quintiles each (table 1). In the following, the terms responder and non-responder were used according to the different response in ISI, albeit non-responders showed changes in other parameters post-intervention (table 1). The HRE group had an average 1.6-fold increase in ISI, while the NRE group had even a small decrease of ISI (0.9-fold) after the exercise intervention (Fig. S1). A significant interaction of response group and intervention was found for ISI, $glucose_{120min}$ and plasma triglycerides (table 1, p1-value). IAT was increased similarly in both groups (table 1). Pre-intervention parameters, e.g. insulin sensitivity, fitness and age were not different between the groups (table 1, p2-value) and both groups trained with the same intensity (HRE vs. NRE, cycling: 0.33±0.11 vs. 0.31±0.09 kJ/kg/min; p=0.8; treadmill: 0.39±0.10 vs. 0.35±0.06 kJ/kg/min; p=0.5).

Impaired up-regulation of mitochondrial energy metabolism genes in ISI non-responders

Transcriptome analyses of the muscle biopsies taken before and after the eight-weeks training intervention revealed the differential regulation of 469 genes after training comparing the HRE

and NRE groups ($p<0.05$, ratio of fold changes > 1.2 x). Significantly enriched GO terms were dominated by cellular functions and components related to mitochondria and metabolic pathways located in the mitochondrial matrix, in particular fatty acid β-oxidation, tricarboxylic acid cycle, oxidative phosphorylation and respiratory electron transport chain (Fig. 1A). The transcripts of these mitochondrial enzymes and carriers located in the inner mitochondrial membrane showed impaired up-regulation in the NRE group compared to the HRE group, e.g. β-oxidation enzymes carnitine palmitoyltransferase (*CPT1B*) and hydroxyacyl-CoA dehydrogenases (*HADH*) and ATP synthase (*ATP5*). Similarly, glucose transporter 4 (*SLC2A4*) and enzymes of glucose catabolism were not up-regulated in NRE compared to HRE (Fig. 1A), suggesting not only impaired improvement of fatty acid oxidation capacity and ATP production post intervention in NRE, but also reduced up-regulation of glucose uptake and oxidation. Before intervention, abundance of these transcripts were not different between HRE and NRE, except for *CPT1B* (0.82-fold lower in NRE, $p=0.03$).

Ingenuity software was used to search for upstream regulators that could explain the differences in gene regulation between NRE and HRE. The data indicate impaired activation of several regulators of exercise-dependent gene expression in the skeletal muscle of NRE, namely peroxisome proliferator receptor gamma coactivator PGC1α (gene name *PPARGC1*), PPAR transcription factors, estrogen receptors (*ESRRA, ESR)* and AMPKα2 subunit (gene name *PRKAA2*) (Fig. 1B). *PPARGC1A* transcript abundance was slightly increased in HRE after training but reduced in NRE (Fig. 1C). The NRE group also showed a reduced abundance of *PRKAA2* post intervention (Fig. 1C). The fold change in transcripts of *PPARGC1* and *PRKAA2* correlated with the fold change in insulin sensitivity in the entire study group (Fig. 1D,E). Abundance of transcripts was also quantified by qPCR and gave comparable results (table S2

and S3). Protein abundance of AMPKα2 and of the ATP synthase subunit α (ATP5A) was significantly increased in HRE, while the increase was attenuated in NRE (Fig. 1F-H).

Increased up-regulation of immune response and inflammatory genes and TGFβ target genes in ISI non-responders

In addition, GO terms related to immune response, inflammatory processes and toll-like receptor signaling were significantly enriched among differentially regulated genes in the HRE/NRE comparison. These differentially regulated transcripts were up-regulated in the NRE group after the intervention, and unchanged or even reduced in HRE (Fig. 2A,B). The transcripts are known to be highly expressed in macrophages (*CD68*, *CXCR2*, *CD14*, *TLR4*) and neutrophils (*CD97*, *CYBB*, *NCF1*), or act as chemoattractant (*RARRES2*). The data suggest increased infiltration of macrophages and neutrophiles and enhanced inflammation in skeletal muscle of NRE post-intervention, since no difference in the abundance of transcripts was found before intervention when comparing HRE vs. NRE ($p<0.05$, ratio >1.2-fold).

Candidate upstream regulators specifically activated in NRE are mitogen-activated protein kinases, inflammatory cytokines and transforming growth factor TGFβ1 (gene name *TGFB1*) (Fig. 2C). Several TGFβ1 target genes were up-regulated post intervention and showed a negative correlation with the change in insulin sensitivity in the entire group: TGFβ inducible protein *TGFBI,* collagens and matrix metallopeptidases (Fig. 2D-F, Fig. S2A-C). TGFβ1 itself was the only upstream candidate with a differential transcript abundance post intervention; NRE showed slightly increased *TGFB1* abundance compared to HRE (1.25-fold, $p<0.05$). Moreover, increased up-regulation of the TGFβ receptor 2 was associated with ISI non-response (Fig. 2G). These data point to an activation of TGFβ signaling in the muscle of NRE, resulting in altered

regulation of TGFβ target genes. Plasma TGFβ1, also after relation to platelet factor 4, and TGFβ inducible protein levels were not changed after training and not different between HRE and NRE (Fig. 2H-J). Thus it is possible that the different activation of TGFβ signaling is restricted to the skeletal muscle of HRE and NRE.

TGFβ1 suppresses mitochondrial regulators and enzymes in human skeletal muscle

TGFβ1 activity is not only dependent on its protein abundance; after secretion the bioavailability of the active protein is regulated at multiple steps. The increase in the expression of the TGFβ1 inducible protein *TGFBI* showed a strong correlation with the up-regulation of other TGFβ1 target genes in the muscle biopsies, exemplarily shown for *FN1* and *COL1A2* (Fig. S2D,E) and thus behaves as a good readout of active TGFβ1 in skeletal muscle. Notably, the increased expression of the TGFβ1 inducible protein *TGFBI* correlated inversely with the down-regulation of *PPARGC1A* and *PRKAA2* (Fig. 3A,B). Thus, the muscle transcriptome data suggest a negative regulation of *PPARGC1A* and *PRKAA2* expression by TGFβ1.

We tested this hypothesis in human skeletal muscle cells. When differentiated myotubes were incubated with TGFβ1 for 24 h we found a significant decrease in mRNA abundance in *PPARGC1A, PRKAA2*, the mitochondrial transcription factor *TFAM* and for key regulators of β-oxidation, *HADHA* and *CPT1B* (Fig. 3C-G). This decrease was prevented by co-treatment with the TGFβ receptor 1 antagonist SB431543. Treatment with SB431543 alone increased expression of *PPARGC1A* and *CPT1B*. This effect can be due to the inhibition of autocrine TGFβ1 activity, which is also visible as reduced expression of the TGFβ1 target gene *TGFBI* in the presence of SB431543 (Fig. 3H). TGFβ1 treatment of myotubes had also an inhibitory effect on insulin signaling since it reduced insulin-stimulated phosphorylation of Akt/PKB (Fig. 3I-K).

When the cells were treated for 3 or 7 days with TGFβ1, a marked down-regulation of *PPARGC1A* was observed, while treatment with the TGFβ receptor 1 antagonist SB431543 alone increased the expression approximately 5-fold (Fig. 4A). A TGFβ1-induced inhibition was also found for *PRKAA2, TFAM*, *HADHA,* and *CPT1B* expression, with an opposite effect of the antagonist SB431543 (Fig. 4B-E). Moreover, TGFβ1 reduced the protein abundance of AMPKα2 and of ATP5A (Fig. 4F-H). TGFβ1 treatment also blocked the increase in mRNA abundance of *MYH2* and *MYH7* and muscle cell differentiation (Fig. 4I-K). Notably, cells obtained from donors identified as ISI responders and non-responders did not differ in their response to TGFβ1 (Fig. S3). To conclude, these data indicate that TGFβ1 down-regulates the abundance of mitochondrial regulators and mitochondrial enzymes in skeletal muscle cells. This inhibitory effect is found in differentiated myotubes and during cell differentiation.

Discussion

In our study, we provide evidence for a molecular mechanism which can contribute to the failure to improve insulin sensitivity after exercise intervention. Given the enormous relevance of physical activity in prevention and therapy of type 2 diabetes, and a notable quantity of individuals who showed no beneficial effects of training on metabolic parameters (22), it is important to understand the molecular basis of this exercise non-response.

All subjects in our study improved their IAT, but they showed a huge variability in their fold change in insulin sensitivity after the supervised, eight weeks endurance training. We clearly defined a group of responders that could increase their insulin sensitivity after the training intervention and a group of non-responders. Using whole genome microarray analyses of the

muscle biopsies taken before and after intervention we gained unbiased insight into the training-induced molecular changes in muscle of the responders and non-responders.

We identified specific transcriptional profiles in the muscle of the NRE group and provide evidence in cell culture studies for a role of TGFβ1 as one negative regulator of mitochondrial activators and enzymes potentially preventing a beneficial metabolic adaptation to training. The non-responders showed increased expression of TGFβ1, of the receptor *TGFBR2*, and of several target genes of TGFβ. Notably, the transcripts which indicate higher TGFβ activity were not different before intervention. Thus, this different transcriptional profile is caused by a different response to training.

In addition, the failure to improve insulin sensitivity is associated with impaired up-regulation of genes important for glucose and fatty acid oxidation and mitochondrial oxidative phosphorylation. The transcriptional coactivator PGC1α and AMPK were identified as relevant upstream regulators that are implicated in this exercise non-response. Importantly, the mRNA abundance of genes encoding for PGC1α and AMPKα2 was down-regulated in the muscle of non-responders post-intervention. We proved a causal relationship of the increased TGFβ1 activity and the suppression of PGC1α and AMPKα2 in human skeletal muscle cells. TGFβ1 down-regulated and blockade of TGFβ signaling up-regulated the expression of PGC1α and AMPKα2, and consequently of the mitochondrial transcription factor *TFAM,* which is regulated by PGC1α (23). Moreover, TGFβ1 down-regulated key regulators of mitochondrial fatty acid oxidation, what is likely to be a consequence of the negative effect on PGC1α and AMPK. Both proteins are key players in the exercise-dependent regulation of enzymes involved in fuel oxidation (24). Thus, the cell culture data provide clear evidence for the molecular mechanism suggested by the transcriptome analyses of the muscles of responders and non-responders.

On a molecular level, activation of TGFβ1 signaling leads to phosphorylation of SMAD3, which can act as a transcriptional repressor at the PGC1α promoter (25). Importance of the TGFβ/SMAD3 pathway for metabolic control in humans is suggested by a close association of BMI and circulating TGFβ1 levels (26) and the association of elevated plasma TGFβ1 and higher risk for type 2 diabetes (27). A rat model with insulin resistance and a low aerobic response to exercise shows after an acute exercise bout increased activation of SMAD3-dependent gene expression when compared to rats with a high aerobic response (28). Although the causal relationship needs to be proven in vivo, these data point to chronically elevated TGFβ1 activity as negative regulator in the adaptation of fuel oxidation in response to training, demonstrated for the first time in a human exercise intervention study. The TGFβ-mediated inhibition of skeletal muscle differentiation as shown in the human muscle cells in our study and described earlier (29) can play an additional role here.

While all participants in our study were untrained and overweight, only some of them showed an adverse response to the training intervention. The increased expression of markers of macrophages, neutrophils and inflammation suggests local inflammatory processes in the muscle of these non-responders, even though increased recruitment of immune cells was not investigated by immunohistochemistry in our study. Activated resident macrophages and infiltrated immune cells release TGFβ, which is a physiological response of the skeletal muscle to mechanical loading and is involved in the synthesis and reorganization of extracellular matrix components (30). The recruitment of macrophages and neutrophils is often associated with acute eccentric or resistance exercise (31), but it can also occur after endurance exercise (32). TGFβ then activates fibroblasts in the muscle to produce extracellular matrix proteins and TGFβ itself (33), while muscle satellite cells and myofibers may contribute to the enhanced production of TGFβ and

extracellular matrix proteins (34;35). This process possibly takes place in all participants of our study after the first training sessions, as part of the normal regeneration program of an untrained muscle (33;36). Since the training intensity was comparable between the HRE and NRE group, higher intensity cannot explain the increased expression of inflammatory markers and TGFβ target genes in the muscle of NRE. It is more likely that the adaptation process after the unaccustomed physical activity is different. This indicates the importance of carefully designed, individualized training protocols for physically inactive subjects to avoid the possible consequences of unaccustomed skeletal muscle activity. On the other hand, we cannot rule out the possibility that the duration or intensity of our training protocol was not conducive for the metabolic improvement of some of our study participants. Extension of the training period with reduced exercise intensity or a combination of high intensity training and resistance training might be a way to overcome the lack of metabolic improvement. A limitation of our study is the self-determined order of the exercise type (walking/cycling) in each training session. The order was not recorded and might have influenced the training outcome. Moreover, we do not know whether the muscle used for the biopsies was similarly recruited during walking and cycling in all subjects.

Clearly, increased TGFβ activity is only one potential contributor to the individual exercise response. An interesting upstream regulator candidate with a high activation score in the NRE group compared to HRE which we did not further investigate in this study is the mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4). MAP4K4 deficiency in skeletal muscle of mice improves insulin sensitivity and protect from obesity-induced insulin resistance (37) and genetic polymorphisms in the *MAP4K4* locus have been associated with insulin

resistance (38). Thus activation of this kinase in skeletal muscle may be another regulator of the individual differences in the metabolic response to training interventions.

Our data underline the importance of PGC1α for the beneficial effects of exercise on metabolic control. PGC1α is considered as an important upstream regulator of the training-induced metabolic adaptations due to its potency to activate mitochondrial biogenesis and the formation of oxidative muscle fibers (23;39;40). In addition, genetic variations in the *PPARGC1A* gene were associated to the change in insulin sensitivity and fitness after a nine month lifestyle intervention (41). Our results are also well in accordance with a recent study, which shows that only individuals who achieved normalized glucose tolerance after exercise training had increased mRNA expression levels of enzymes of mitochondrial oxidation and their upstream regulators (42), while expression levels remained unaltered in individuals who could not improve their glucose control. Although the intervention protocol in that study differs from our study in several aspects (four-months, non-supervised walking intervention vs. two-months, supervised endurance training), the responders in that study and our HRE group show very similar changes in the expression of metabolic and mitochondrial markers in skeletal muscle.

In conclusion, our data suggest a causal relationship of enhanced TGFβ1 activity in skeletal muscle after training and the suppression of key mitochondrial regulators. The results can contribute to our understanding of the failure of the skeletal muscle to participate in the improvement of insulin sensitivity after exercise intervention. Since skeletal muscle accounts for approximately 30 - 40% of total body weight in non-obese humans and for more than 85% of insulin-dependent glucose uptake (43) this tissue plays a major role in whole body metabolic control. Our data underline the importance of personalized training strategies and can open for

the first time perspectives to target exercise non-response by the prevention of dysregulated TGFβ1 signaling in skeletal muscle.

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Author contributions

Conceptualization, A.B., A.F., N.S., A.N., H.U.H., and C.W.; Investigation, A.B., C.H., M.I., P.S., G.S., C.S., V.S., J.H., and J.M.; Formal Analysis, A.B., C.H., M.I., J.M., and C.W.; Writing – Original Draft, A.B. C.H., M.I., and C.W.; Writing –Review & Editing, J.B., A.N., H.U.H., and C.W.; Funding Acquisition, A.B., J.B., M.H.A, H.U.H., and C.W.; Resources, F.S., J.B., and A.N.; Supervision, F.S., J.B., M.H.A., H.S., A.F., N.S., A.N., H.U.H., and C.W.

C.W. 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.

Duality of interest No potential conflicts of interest relevant to this article were reported.

Data and materials availability: Array data has been submitted to GEO (GSE72462).

References

1. Boule NG, Weisnagel SJ, Lakka TA, Tremblay A, Bergman RN, Rankinen T, Leon AS, Skinner JS, Wilmore JH, Rao DC, Bouchard C: Effects of exercise training on glucose homeostasis: the HERITAGE Family Study. Diabetes Care 2005;28:108-114

2. Praet SF, van Loon LJ: Exercise: the brittle cornerstone of type 2 diabetes treatment. Diabetologia 2008;51:398-401

3. Hawley JA: Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. Diabetes Metab Res Rev 2004;20:383-393

4. Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, Uusitupa M: Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 2001;344:1343-1350

5. Abouassi H, Slentz CA, Mikus CR, Tanner CJ, Bateman LA, Willis LH, Shields AT, Piner LW, Penry LE, Kraus EA, Huffman KM, Bales CW, Houmard JA, Kraus WE: The effects of aerobic, resistance, and combination training on insulin sensitivity and secretion in overweight adults from STRRIDE AT/RT: a randomized trial. J Appl Physiol (1985) 2015;118:1474-1482

6. Richter EA, Turcotte L, Hespel P, Kiens B: Metabolic responses to exercise. Effects of endurance training and implications for diabetes. Diabetes Care 1992;15:1767-1776

7. Stephens NA, Sparks LM: Resistance to the Beneficial Effects of Exercise in Type 2 Diabetes: Are Some Individuals Programmed to Fail? J Clin Endocrinol Metab 2015;100:43-52

8. Bouchard C, Blair SN, Church TS, Earnest CP, Hagberg JM, Hakkinen K, Jenkins NT, Karavirta L, Kraus WE, Leon AS, Rao DC, Sarzynski MA, Skinner JS, Slentz CA, Rankinen T: Adverse metabolic response to regular exercise: is it a rare or common occurrence? PLoS One 2012;7:e37887

9. Sparks LM, Johannsen NM, Church TS, Earnest CP, Moonen-Kornips E, Moro C, Hesselink MK, Smith SR, Schrauwen P: Nine months of combined training improves ex vivo skeletal muscle metabolism in individuals with type 2 diabetes. J Clin Endocrinol Metab 2013;98:1694-1702

10. Church TS, Blair SN, Cocreham S, Johannsen N, Johnson W, Kramer K, Mikus CR, Myers V, Nauta M, Rodarte RQ, Sparks L, Thompson A, Earnest CP: Effects of aerobic and resistance training on hemoglobin A1c levels in patients with type 2 diabetes: a randomized controlled trial. JAMA 2010;304:2253-2262

11. Bajpeyi S, Tanner CJ, Slentz CA, Duscha BD, McCartney JS, Hickner RC, Kraus WE, Houmard JA: Effect of exercise intensity and volume on persistence of insulin sensitivity during training cessation. J Appl Physiol (1985) 2009;106:1079-1085

12. Lakka TA, Rankinen T, Weisnagel SJ, Chagnon YC, Rice T, Leon AS, Skinner JS, Wilmore JH, Rao DC, Bouchard C: A quantitative trait locus on 7q31 for the changes in plasma insulin in response to exercise training: the HERITAGE Family Study. Diabetes 2003;52:1583-1587

13. Ruchat SM, Rankinen T, Weisnagel SJ, Rice T, Rao DC, Bergman RN, Bouchard C, Perusse L: Improvements in glucose homeostasis in response to regular exercise are influenced by the PPARG Pro12Ala variant: results from the HERITAGE Family Study. Diabetologia 2010;53:679-689

14. Matsuda M, DeFronzo RA: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 1999;22:1462-1470

15. Machann J, Thamer C, Schnoedt B, Haap M, Haring HU, Claussen CD, Stumvoll M, Fritsche A, Schick F: Standardized assessment of whole body adipose tissue topography by MRI. J Magn Reson Imaging 2005;21:455-462

16. Roecker K, Schotte O, Niess AM, Horstmann T, Dickhuth HH: Predicting competition performance in long-distance running by means of a treadmill test. Med Sci Sports Exerc 1998;30:1552-1557

17. Peronnet F, Massicotte D: Table of nonprotein respiratory quotient: an update. Can J Sport Sci 1991;16:23-29

18. Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Haring HU, Schleicher ED: Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. J Biol Chem 2004;279:23942-23952

19. Wakefield LM, Letterio JJ, Chen T, Danielpour D, Allison RS, Pai LH, Denicoff AM, Noone MH, Cowan KH, O'Shaughnessy JA: Transforming growth factor-beta1 circulates in normal human plasma and is unchanged in advanced metastatic breast cancer. Clin Cancer Res 1995;1:129-136

20. Rainer J, Sanchez-Cabo F, Stocker G, Sturn A, Trajanoski Z: CARMAweb: comprehensive R- and bioconductor-based web service for microarray data analysis. Nucleic Acids Res 2006;34:W498-W503

21. Eichner J, Rosenbaum L, Wrzodek C, Haring HU, Zell A, Lehmann R: Integrated enrichment analysis and pathway-centered visualization of metabolomics, proteomics, transcriptomics, and genomics data by using the InCroMAP software. J Chromatogr B Analyt Technol Biomed Life Sci 2014;966:77-82

22. Stephens NA, Sparks LM: Resistance to the Beneficial Effects of Exercise in Type 2 Diabetes: Are Some Individuals Programmed to Fail? J Clin Endocrinol Metab 2014;jc20142545

23. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM: Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115-124

24. Egan B, Zierath JR: Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab 2013;17:162-184

25. Tiano JP, Springer DA, Rane SG: SMAD3 Negatively Regulates Serum Irisin and Skeletal Muscle FNDC5 and Peroxisome Proliferator-activated Receptor gamma Coactivator 1-alpha (PGC-1alpha) during Exercise. J Biol Chem 2015;290:7671-7684

26. Yadav H, Quijano C, Kamaraju AK, Gavrilova O, Malek R, Chen W, Zerfas P, Zhigang D, Wright EC, Stuelten C, Sun P, Lonning S, Skarulis M, Sumner AE, Finkel T, Rane SG: Protection from obesity and diabetes by blockade of TGF-beta/Smad3 signaling. Cell Metab 2011;14:67-79

27. Herder C, Zierer A, Koenig W, Roden M, Meisinger C, Thorand B: Transforming growth factor-beta1 and incident type 2 diabetes: results from the MONICA/KORA case-cohort study, 1984-2002. Diabetes Care 2009;32:1921-1923

28. Lessard SJ, Rivas DA, Alves-Wagner AB, Hirshman MF, Gallagher IJ, Constantin-Teodosiu D, Atkins R, Greenhaff P, Qi NR, Gustafsson T, Fielding RA, Timmons JA, Britton SL, Koch LG, Goodyear LJ: Resistance to aerobic exercise training causes metabolic dysfunction and reveals novel exercise-regulated signaling networks. Diabetes 2013;62:2717-2727

29. Massague J, Cheifetz S, Endo T, Nadal-Ginard B: Type beta transforming growth factor is an inhibitor of myogenic differentiation. Proc Natl Acad Sci U S A 1986;83:8206-8210

30. Kjaer M: Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol Rev 2004;84:649-698

31. Paulsen G, Mikkelsen UR, Raastad T, Peake JM: Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc Immunol Rev 2012;18:42-97

32. Neubauer O, Sabapathy S, Ashton KJ, Desbrow B, Peake JM, Lazarus R, Wessner B, Cameron-Smith D, Wagner KH, Haseler LJ, Bulmer AC: Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling. J Appl Physiol (1985) 2014;116:274-287

33. Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Munoz-Canoves P: Aberrant repair and fibrosis development in skeletal muscle. Skelet Muscle 2011;1:21

34. Alexakis C, Partridge T, Bou-Gharios G: Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. Am J Physiol Cell Physiol 2007;293:C661-C669

35. Weigert C, Lehmann R, Hartwig S, Lehr S: The secretome of the working human skeletal muscle--a promising opportunity to combat the metabolic disaster? Proteomics Clin Appl 2014;8:5-18

36. Burks TN, Cohn RD: Role of TGF-beta signaling in inherited and acquired myopathies. Skelet Muscle 2011;1:19

37. Danai LV, Flach RJ, Virbasius JV, Menendez LG, Jung DY, Kim JH, Kim JK, Czech MP: Inducible Deletion of Protein Kinase Map4k4 in Obese Mice Improves Insulin Sensitivity in Liver and Adipose Tissues. Mol Cell Biol 2015;35:2356-2365

38. Sartorius T, Staiger H, Ketterer C, Heni M, Machicao F, Guilherme A, Grallert H, Schulze MB, Boeing H, Stefan N, Fritsche A, Czech MP, Haring HU: Association of common genetic variants in the MAP4K4 locus with prediabetic traits in humans. PLoS One 2012;7:e47647

39. Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, Lebrasseur NK, Yan Z, Spiegelman BM: Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. J Biol Chem 2007;282:30014-30021

40. Pilegaard H, Saltin B, Neufer PD: Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 2003;546:851-858

41. Stefan N, Thamer C, Staiger H, Machicao F, Machann J, Schick F, Venter C, Niess A, Laakso M, Fritsche A, Haring HU: Genetic variations in PPARD and PPARGC1A determine mitochondrial function and change in aerobic physical fitness and insulin sensitivity during lifestyle intervention. J Clin Endocrinol Metab 2007;92:1827-1833

42. Osler ME, Fritz T, Caidahl K, Krook A, Zierath JR, Wallberg-Henriksson H: Changes in Gene Expression in Responders and Nonresponders to a Low-Intensity Walking Intervention. Diabetes Care 2015;38:1154-1160

43. DeFronzo RA, Ferrannini E, Sato Y, Felig P, Wahren J: Synergistic interaction between exercise and insulin on peripheral glucose uptake. J Clin Invest 1981;68:1468-1474

Figure legends

Fig. 1
Impaired up-regulation of mitochondrial energy metabolism genes in ISI non-responders
(**A**) Transcriptome analysis revealed differentially regulated genes in muscle biopsies of non-responders vs. high responders after exercise related to glucose and fatty acid (FA) catabolism, amino acid metabolism, tricarboxylic acid cycle (TCA) or oxidative phosphorylation (ratio of fold changes in the comparison NRE/HRE post-intervention vs. pre-intervention; $p<0.05$). (**B**) Upstream regulators less activated (inhibited) in NRE vs. HRE based on Ingenuity upstream regulator analysis of differentially regulated transcripts. Z-scores below -2 can be considered significant. (**C**) Fold change post-intervention vs. pre-intervention of *PPARGC1A*, and *PRKAA2* transcripts (transcriptome data) in muscle biopsies of the participants grouped into high responders (HRE, n=7), an intermediate group (I, n=4) and non-responders (NRE, n=7) (table 1); bar graphs illustrate the mean of each group; * $p< 0.05$ fold change NRE vs. fold change HRE. (**D**,**E**) Correlation of log-transformed fold changes in ISI and fold changes in transcripts (n=18). (**F-H**) Immunoblots of protein lysates of muscle biopsies using indicated antibodies. Detection of GAPDH serves as loading control of separate blots. Relative band intensity values normalised to GAPDH are shown (n=4-7, mean±SD; * $p<0.05$ vs. pre; # $p<0.05$ HRE post vs. NRE post). Lanes between pre- and post-samples and between the samples and the molecular weight (MW) marker were empty. The lane marked with arrow does not show any detectable ATP5α or AMPKα2 signal, hence it was excluded from quantification.

Fig. 2
Increased up-regulation of immune and inflammatory genes and TGFβ target genes in ISI non-responders
Transcriptome analysis revealed differentially regulated genes in high responders vs. non-responders after exercise related to immune response and inflammatory processes (**A**) and toll-like receptor signaling (**B**). Shown is the fold change post-intervention vs. pre-intervention of transcripts in muscle biopsies of the participants grouped into high responders (HRE, n=7), an intermediate group (I, n=4) and non-responders (NRE, n=7) (table 1); bar graphs illustrate the mean of each group; * $p<0.05$ fold change NRE vs. fold change HRE. (**C**) Upstream regulators activated in NRE vs. HRE based on Ingenuity upstream regulator analysis of differentially regulated transcripts. Z-scores greater than 2 can be considered significant. (**D-G**) Correlation of log-transformed fold changes in ISI and fold changes (post-intervention vs. pre-intervention) in TGFβ target gene and *TGFBR2* transcripts in muscle biopsies assessed by transcriptome analyses (n=18). (**H**) Plasma concentration of TGFβ1, (**I**) TGFβ1 related to plasma concentration of platelet factor 4 (PF4), and (**J**) TGFβ-inducible protein (TGFBI) in samples of high responders (HRE, n=8) and non-responders (NRE, n=8) before (pre) and after eight weeks training intervention (post). Individual concentrations are shown as black dots, bar graphs illustrate the mean ± SD of each group.

Fig. 3
TGFβ1 activity is related to suppression of mitochondrial regulators in human skeletal muscle
(**A**,**B**) Correlation of log-transformed fold changes (post-intervention vs. pre-intervention) in *PPARGC1A* or *PRKAA2* and *TGFBI* transcripts in muscle biopsies assessed by transcriptome

analyses (n=18). (**C-H**) Relative fold change of mRNA abundance of indicated genes related to *TBP* measured by qPCR in fully differentiated human skeletal muscle cells treated with 10µM SB 431524, 2ng/ml TGFβ1, or both (TGF+SB) for 24 h. Shown is the fold change compared with vehicle-treated cells (n=4, mean±SD); * $p<0.05$ vs. vehicle-treated cells; #$p<0.05$ vs. TGFβ1-treated cells). **(I-K)** Human skeletal muscle cells were treated with 1 ng/ml TGFβ1 or 10 µM SB431524 for 24 h before stimulation with 10 nM insulin for 10 min. Representative immunoblots of protein lysates (in duplicate) using the antibodies as indicated and relative band intensity values related to AKT were shown (n=3-6, mean±SD; * $p<0.05$ vs. vehicle-treated cells; # $p<0.05$ vs. insulin-treated cells).

Fig. 4

TGFβ1 suppresses mitochondrial regulators and enzymes in human skeletal muscle cells

Human skeletal muscle cells were treated with 0.2 or 1ng/ml TGFβ1 or 10µM SB431524 for 3 or 7 days. (**A-E;I,J**) Relative fold change of mRNA abundance of indicated genes related to *TBP* measured by qPCR. Shown is the fold change compared with vehicle-treated cells (n=4, mean±SD; * $p<0.05$ vs. vehicle-treated cells). (**F-H**) Representative immunoblots of protein lysates (in duplicate) using the indicated antibodies. Detection of RPS6 serves as loading control of separate blots. Relative band intensity values normalized to RPS6 are shown as fold change compared with vehicle-treated cells (n=4, mean±SD; * $p<0.05$ vs. vehicle-treated cells) (**K**) Immunostaining of CD56, MYH2 (fast) and MYH7 (slow) in human skeletal muscle cells treated with 1ng/ml TGFβ1 or TGFβ1 and 10µM SB431524 for 7 days. Nuclei are shown in red.

Table 1

	High responder (HRE, n=8 [6f, 2m])			Intermediate group (n=4 [2f, 2m])			Non responder (NRE, n=8 [5f, 3m])			HRE vs.NRE	
	pre	post	p	pre	post	p	pre	post	p	time*group p1	pre p2
ISI OGTT (10^6 $Lkg^{-1}min^{-1}$)	7.1 ± 6.8	11.8 ± 11.0	0.002	7.8 ± 1.3	7.8 ± 1.0	0.8	9.8 ± 7.7	8.0 ± 5.6	0.016	<0.0001	0.4
IAT bike (W/kg)	1.1 ± 0.4	1.3 ± 0.4	0.004	1.2 ± 0.3	1.4 ± 0.4	0.007	1.1 ± 0.2	1.2 ± 0.3	0.0006	0.9	1.0
IAT tread. (W/kg)	0.9 ± 0.3	1.0 ± 0.3	0.004	1.1 ± 0.3	1.2 ± 0.3	0.07	0.9 ± 0.1	1.0 ± 0.1	0.003	**0.5**	0.6
VO_2peak bike (ml/min)	2.04 ± 0.68*	2.25 ± 0.51	0.09	2.37 ± 0.53	2.54 ± 0.35‡	0.5	2.26 ± 0.62	2.42 ± 0.74*	0.5	0.4	0.9
VO_2peak bike (ml/min/kg)	22.3 ± 7.0*	26.1 ± 6.8	0.09	24.4 ± 4.3	25.7 ± 2.9‡	0.5	22.7 ± 3.9	24.4 ± 5.9*	0.5	0.4	0.7
VO2peak tread. (ml/min)	2.2 ± 0.7	2.3 ± 0.5	0.3	3.0 ± 0.3	2.3 ± 0.2§	-	2.5 ± 0.5	2.6 ± 0.7	0.2	1.0	0.3
VO2peak tread. (ml/min/kg)	25.2 ± 7.2	26.9 ± 6.7	0.3	30.7 ± 2.2	25.2 ± 1.0§	-	25.3 ± 3.5	27.0 ± 4.7	0.1	0.9	0.8
Age (years)	51.6±10.0	51.6±10.0	-	41.0±10.7	41.0±10.7	-	43.8±11.2	43.8±11.2	-	-	0.2
BMI (kg/m^2)	32.0 ± 6.0	31.6 ± 6.1	0.05	31.6 ± 1.9	31.5 ± 2.1	0.8	33.4 ± 4.6	33.1 ± 4.8	0.3	0.7	0.6
Total adipose tissue (kg)	33 ± 12	32 ± 12	0.05	35 ± 5	34 ± 5	0.4	38 ± 12*	38 ± 12*	0.4	0.3	0.4
Visceral adipose tissue (kg)	3.8 ± 0.7	3.6 ± 1.0	0.2	5.1 ± 2.4	5 ± 2.1	0.7	5.6 ± 4.7*	5.4 ± 4.6*	0.7	0.4	1.0
Lean Body Mass (kg)	53.7 ±12.6*	55.7 ±13.3†	0.4	69.6 ±19.5	63.6 ±13.6‡	0.3	60.2 ±18.8	60.7 ±19.0	0.3	0.9	0.5
RR_{sys} (mmHg)	130 ± 18	130 ± 8	0.9	141 ± 14	135 ± 21	0.3	139 ± 16	129 ±1 0	0.024	0.1	0.2
RR_{dia} (mmHg)	92 ± 11	91 ± 8	0.8	92 ± 12	88 ± 12	0.4	92 ± 10	84 ± 8	0.017	0.1	1.0
Heart rate (min^{-1})	80 ± 14	70 ± 6	0.038	68 ± 11	70 ± 15	0.4	76 ± 11	71 ± 10	0.1	0.4	0.6
$Glucose_0$ (mmol/L)	5.6 ± 0.5	5.6 ± 0.6	0.8	5.4 ± 0.3	5.5 ± 0.5	0.4	5.7 ± 0.5	5.8 ± 0.6	0.4	0.4	0.8
$Glucose_{120}$ (mmol/L)	7.1 ± 1.4	6.0 ± 1.1	0.023	6.1 ± 0.6	5.6 ± 0.6	0.043	6.2 ± 0.7	7.0 ± 1.1	0.09	0.0037	0.1
Free fatty acids (µmol/L)	719 ± 200*	532 ± 195	0.1	469 ± 171	517 ± 169	0.7	550 ± 79	590 ± 149	0.6	0.1	0.1
Triglycerides (mg/dL)	114 ± 39	81 ± 31	0.0004	135 ± 21	118 ± 53	0.5	103 ± 37	105 ± 32	0.6	0.0003	0.6
HDLcholesterol(mg/dL)	49 ± 12	49 ± 11	0.8	37 ± 8	41 ± 5	0.2	50 ± 10	48 ± 11	0.038	0.1	0.8
LDLcholesterol (mg/dL)	118 ± 22	108 ± 19	0.021	104 ± 21	103 ± 29	0.6	124 ± 35	120 ± 29	0.4	0.2	0.8
Leukocytes (μL^{-1})	5944 ± 934	5904 ± 2076	0.6	6190 ± 1767	6045 ± 1454	0.8	6575 ± 2412	6180 ± 1528	0.6	0.9	0.7
CRP (mg/dL)	0.3 ± 0.2	0.6 ± 1.1	0.3	0.1 ± 0.1	0.1 ± 01	0.6	0.2 ± 0.3	0.4 ± 0.5	0.2	0.6	0.5

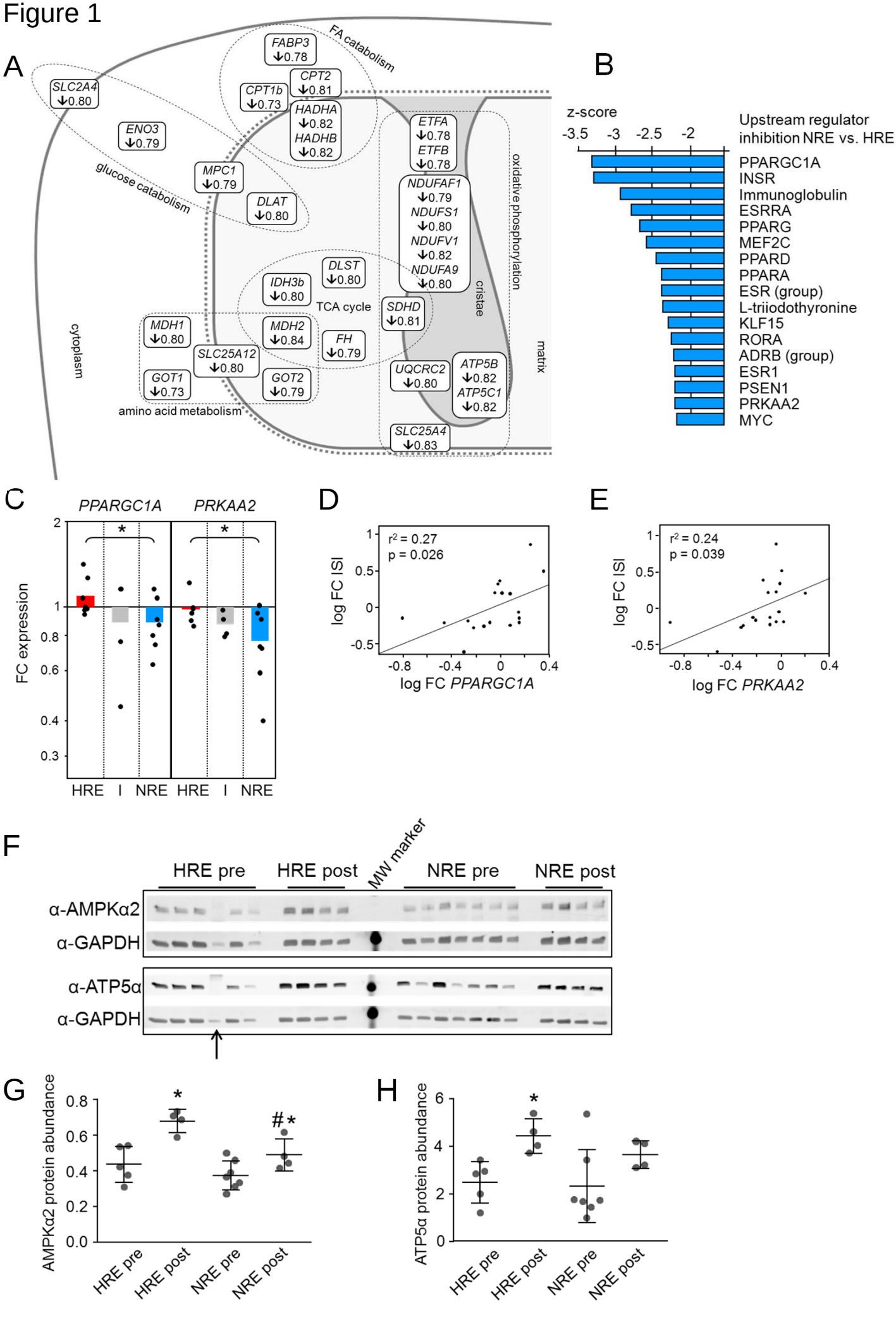
Table 1. Participants' parameters pre and post intervention. Data represent means ±SD. Prior to statistical analysis, data were log_e-transformed in order to approximate normal distribution; p: paired t-test; p1: mANOVA time*group; p2: two-sided t-test, pre-values high responder versus non-responder; * from 7 subjects available; † from 6 subjects available; ‡ from 3 subjects available; § from 2 subjects available; f – female, m – male, ISI – Insulin sensitivity index, IAT – individual anaerobe threshold; RR - blood pressure.

Table 2

Gene symbol	Catalogue number or sequence
COL1A2	for: CTC CAA GGA CAA GAA ACA CGT C rev: ATG TTC TGA GAG GCA TAG TTG GC
CD68	QT00037184
CPT1B	QT00057036
HADHA	QT00091721
MYH2	QT00082495
MYH7	QT00000602
PPARGC1A	QT00095578
PRKAA2	QT00042077
SLC2A4	QT00097902
TBP	QT00000721
TFAM	QT00012782
TGFBI	QT01009477

Table 2. Primer used for quantitative real-time PCR. QT, Quantitect Primer Assays (Qiagen, Hilden, Germany. Primers for COL1A2 were designed in house.

Figure 1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MPC1 ↓0.79
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DLST ↓0.80
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ATP5B ↓0.82
ATP5C1 ↓0.82
SLC25A4 ↓0.83
B
z-score
-3.5 -3 -2.5 -2
Upstream regulator inhibition NRE vs. HRE
PPARGC1A
INSR
Immunoglobulin
ESRRA
PPARG
MEF2C
PPARD
PPARA
ESR (group)
L-triiodothyronine
KLF15
RORA
ADRB (group)
ESR1
PSEN1
PRKAA2
MYC
C
PPARGC1A
PRKAA2
FC expression
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p = 0.026
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log FC PPARGC1A
E
r² = 0.24
p = 0.039
log FC ISI
log FC PRKAA2
F
HRE pre
HRE post
MW marker
NRE pre
NRE post
α-AMPKα2
α-GAPDH
α-ATP5α
α-GAPDH
G
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NRE post
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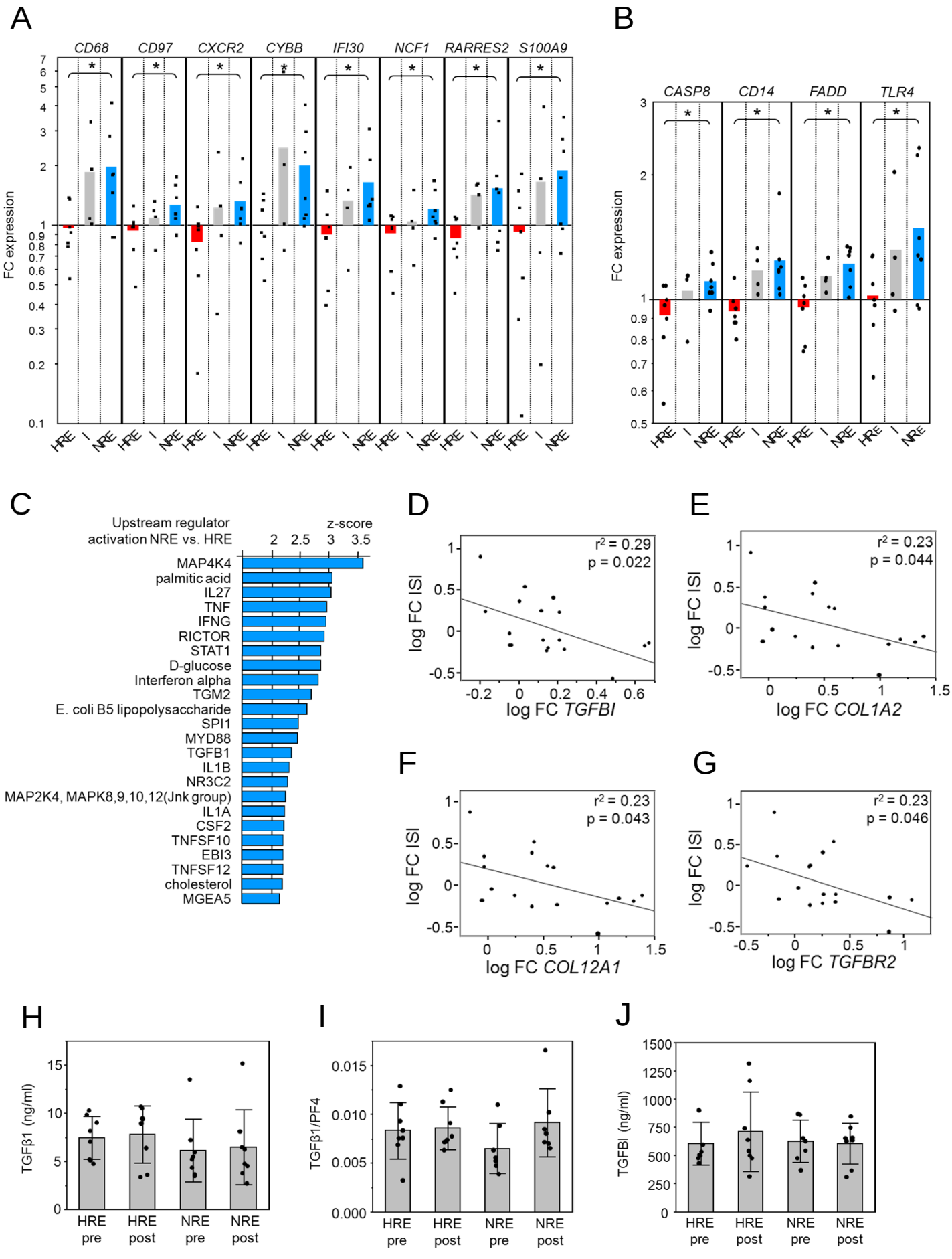
HRE pre
HRE post
NRE pre
NRE post

Figure 2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IL1A
CSF2
TNFSF10
EBI3
TNFSF12
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MGEA5
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p = 0.043
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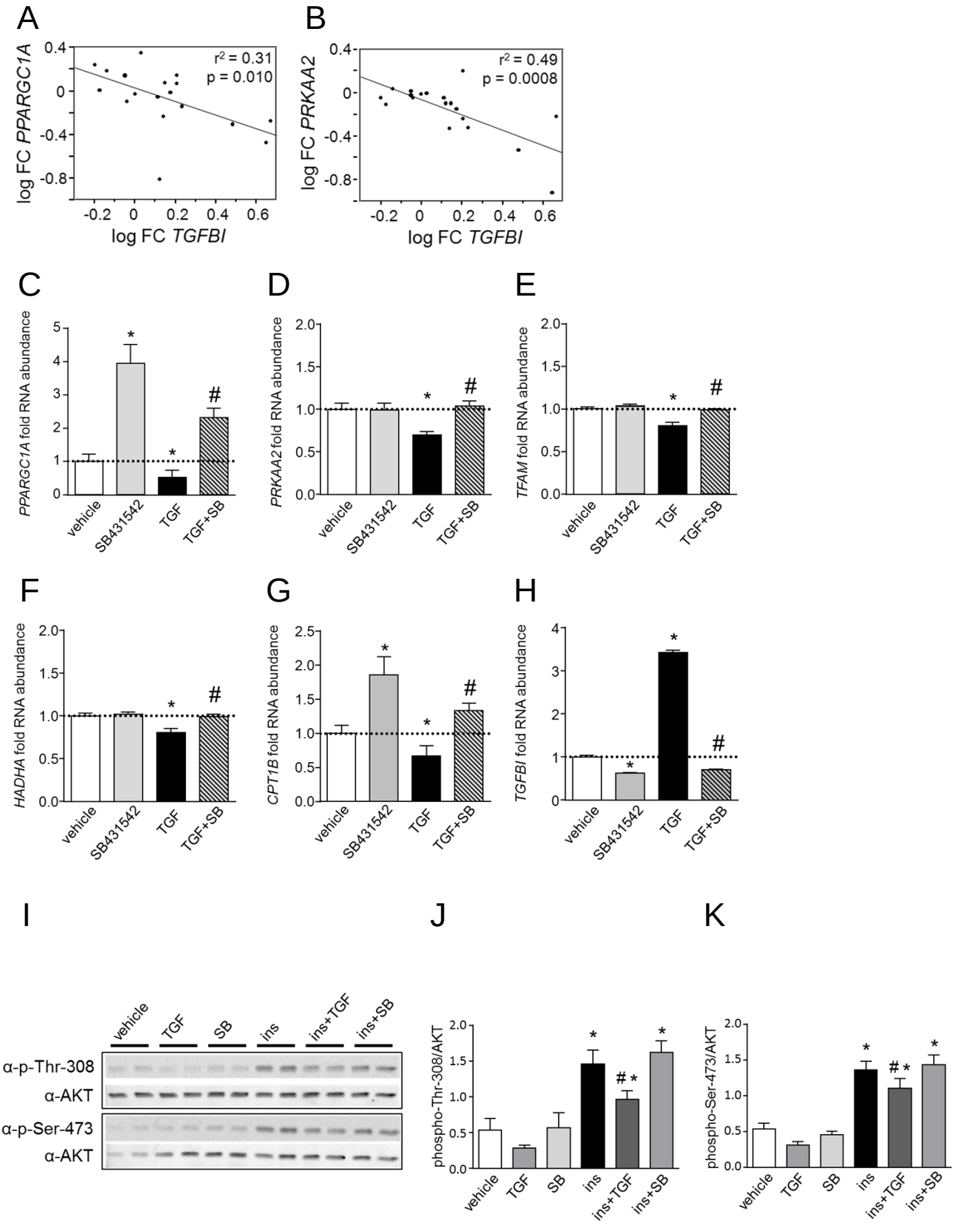
I
TGFβ1/PF4
J
TGFBI (ng/ml)

Figure 3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CPT1B fold RNA abundance
TGFBI fold RNA abundance
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J
K
vehicle
TGF
SB
ins
ins+TGF
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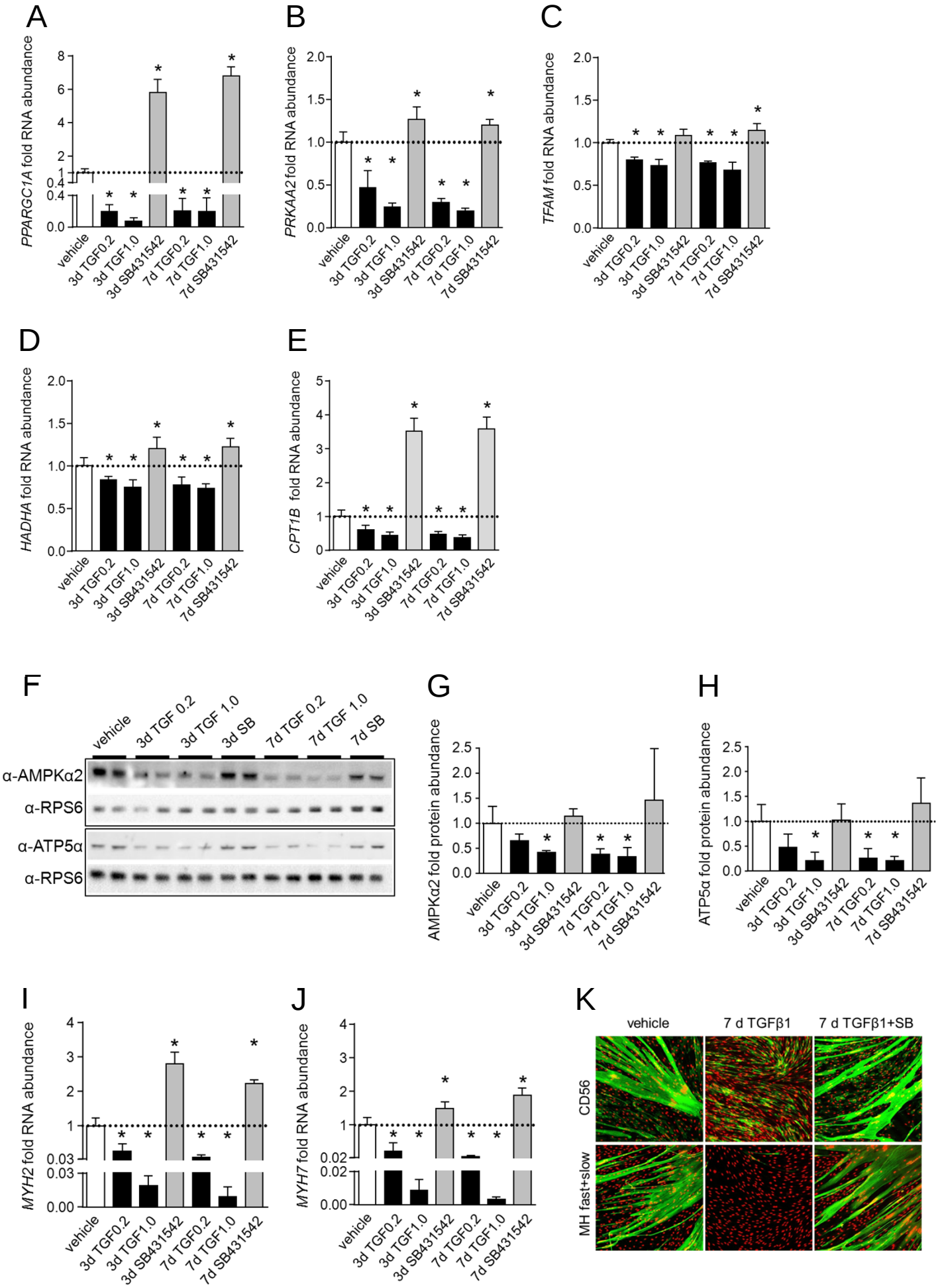
Figure 4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7d TGF 1.0
7d SB
α-AMPKα2
α-RPS6
α-ATP5α
α-RPS6
G
AMPKα2 fold protein abundance
H
ATP5α fold protein abundance
I
MYH2 fold RNA abundance
J
MYH7 fold RNA abundance
K
vehicle
7 d TGFβ1
7 d TGFβ1+SB
CD56
MH fast+slow

Supplemental Data

Figure S1

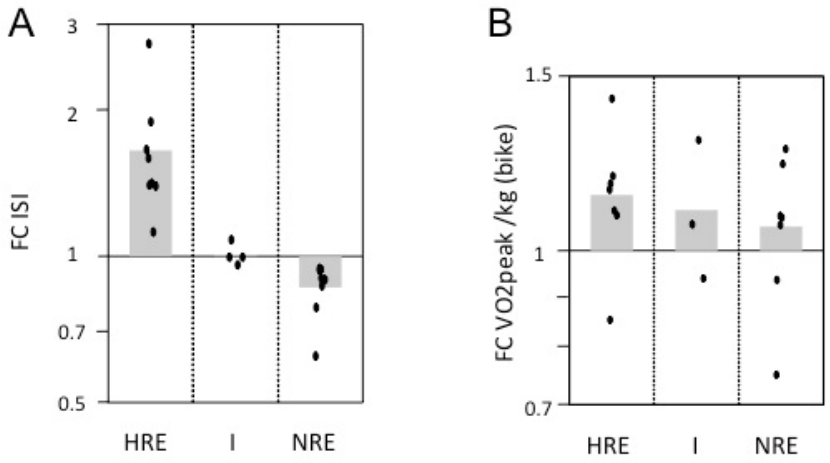

Figure S1

Individual fold changes in insulin sensitivity and VO_2peak

(**A, B**) Insulin sensitivity (ISI) was assessed by the OGTT-based surrogate marker Matsuda index and subjects were grouped into quintiles with 4 in each quintile on the basis of the fold change in ISI Matsuda index. The two upper quintiles with the highest fold change were classified as responders (HRE), the two lower quintiles as non-responders (NRE), and the intermediate quintile as intermediate responders (I). Shown are the individual fold changes (FC) in ISI and in VO_2peak (bike).

Figure S2

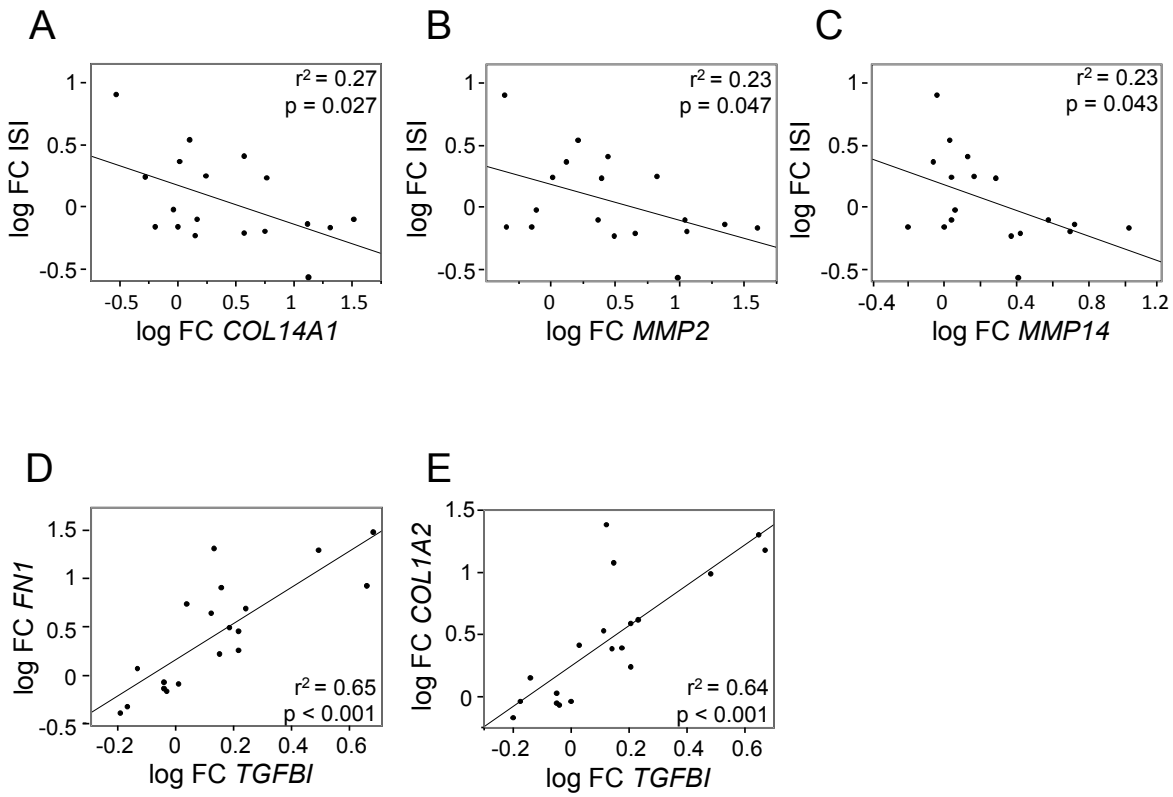

Figure S2

Increased TGFβ target gene expression in muscle of ISI non-responders

(**A**-**C**) Correlation of log-transformed fold changes in ISI and fold changes (post-intervention vs. pre-intervention) in TGFβ target gene expression (transcriptome data of muscle biopsies; n=18). (**D**,**E**) Correlation of log-transformed fold changes (post intervention vs. pre-intervention) in *TGFBI* and *FN1* (**D**) and *COL1A2* (**E**) (transcriptome data of muscle biopsies; n=18).

Figure S3

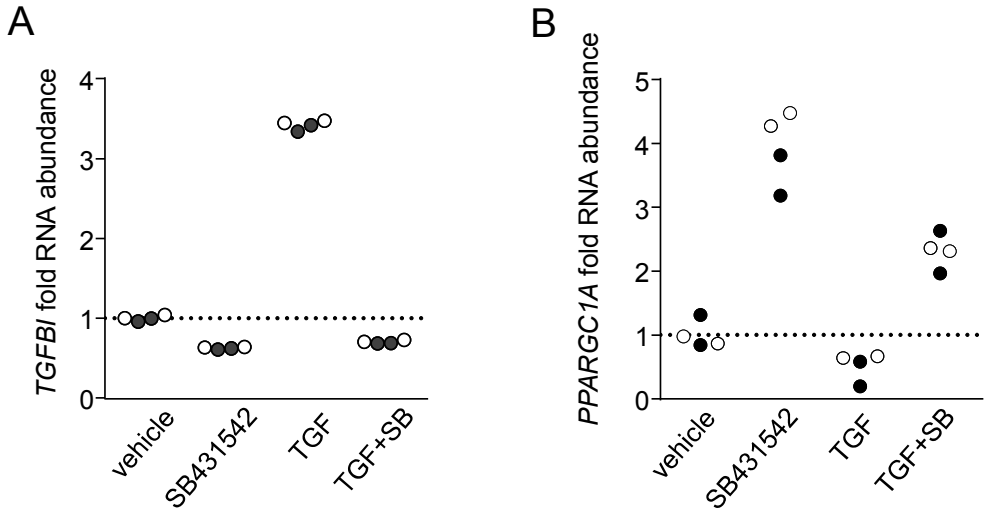

Figure S3

TGFβ1-dependent gene expression in myotubes obtained from HRE and NRE donors

Relative fold change of RNA abundance of *TGFBI* (A) and PPARGC1A (B) measured by qPCR in fully differentiated human skeletal muscle cells treated with 10μM SB 431524, 2ng/ml TGFβ1, or both (TGF+SB) for 24 h. Shown is the fold change compared with vehicle-treated cells of 4 different myotube cultures obtained from 2 HRE (black bowls) and 2 NRE donors (white balls); mean±SD is shown in Fig. 3.

Table S1

	n=20 (13f, 7m)		
	pre	post	p
ISI OGTT ($\cdot 10^6$ $Lkg^{-1}min^{-1}$)	8.3 ± 6.4	9.5 ± 7.8	0.1
IAT bike (W/kg)	1.1±0.3	1.3 ± 0.3	<0.0001
IAT treadmill (W/kg)	0.9±0.2	1.0 ± 0.3	<0.0001
VO_2peak bike (ml/min/kg)	22.9 ± 5.1*	25.4 ± 5.8†	0.047
VO_2peak treadmill (ml/min/kg)	26.3 ± 5.4	26.7 ± 5.3†	0.3
Age (years)	46.4 ± 11.0	46.4± 11.0	-
BMI (kg/m^2)	32.5 ± 4.7	32.2 ± 4.8	0.031
Total adipose tissue (kg)	35.4 ± 10.4*	34.6 ± 10.6*	0.023
Visceral adipose tissue (kg)	4.6 ± 2.8*	4.4 ± 2.8*	0.1
Lean Body Mass (kg)	59.8 ± 17*	59.5 ± 15.7‡	0.1
RR_{sys} (mmHg)	136 ± 16	130 ± 11	0.09
RR_{dia} (mmHg)	92 ± 10	88 ± 9	0.032
Heart rate (min^{-1})	76 ± 13	70 ± 9	0.020
$Glucose_0$ (mmol/L)	5.6 ± 0.5	5.7 ± 0.6	0.5
$Glucose_{120}$ (mmol/L)	6.5 ± 1.1	6.3 ± 1.1	0.4
Free fatty acids (µmol/L)	709 ± 539	552 ± 167	0.2
Triglycerides (mg/dL)	114 ± 36	98 ± 38	0.023
HDL cholesterol (mg/dL)	47 ± 11	47 ± 10	0.9
LDL cholesterol (mg/dL)	117 ± 28	111 ± 25	0.020
Leukocytes (μL^{-1})	6246 ± 1744	6043 ± 1673	0.4
CRP (mg/dL)	0.2 ± 0.2	0.4 ± 0.7	0.1

Table S1. Participants' parameters pre and post intervention. Data represent means ±SD. Prior to statistical analysis, data were $\log_e$-transformed in order to approximate normal distribution; p: paired t-test, *available from 19 subjects; †available from 18 subjects; ‡ available from 17 subjects; f – female, m – male, CRP – C-reactive protein, ISI – Insulin sensitivity index, IAT – individual anaerobic threshold, RR – blood pressure.

Table S2

transcript	r^2	p-value
PPARGC1A	0.74	<0.0001
PRKAA2	0.82	<0.0001
CPT1B	0.80	<0.0001
SLC2A4	0.70	<0.0001
CD68	0.69	<0.0001
TGFBI	0.57	0.0003
COL1A2	0.79	<0.0001

Table S2. Correlation of log-transformed fold changes (post-intervention vs. pre-intervention) in transcript abundance determined by microarray analysis and by qPCR. Data obtained by qPCR are related to transcript abundance of *TBP*.

Table S3

	Microarray data		qPCR data	
transcript	r^2	p-value	r^2	p-value
PPARGC1A	0.27	0.026	0.35	0.01
PRKAA2	0.24	0.039	0.22	0.047
CPT1B	0.57	0.0003	0.40	0.005
SLC2A4	0.42	0.004	0.22	0.048
CD68	0.34	0.01	0.21	0.059
TGFBI	0.29	0.02	0.12	0.15
COL1A2	0.23	0.044	0.15	0.11

Table S3. Comparison of the correlation of log-transformed fold changes in ISI and fold changes in transcript abundance determined by microarray analysis or by qPCR. Data obtained by qPCR are related to transcript abundance of *TBP*.