Original Article

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Browning formation markers of subcutaneous adipose tissue in relation to resting energy expenditure, physical activity and diet in humans

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Abstract:
Background: Regular exercise and diet may contribute to white adipose tissue (WAT) conversion into a brown adipose-like phenotype that may increase resting energy expenditure (REE), leading to weight loss. We examined the relationship between REE, physical activity (PA) participation and diet with browning formation markers of subcutaneous WAT in healthy men.
Materials and methods: We assessed REE, diet and body composition of 32 healthy men [age (years): 36.06 ± 7.36, body mass index (BMI): 27.06 ± 4.62 (kg/m^2)]. Participants also underwent measurements of PA [metabolic equivalent (MET)-min/week] using the International Physical Activity Questionnaire (IPAQ), while they undertook a subcutaneous fat biopsy from the abdominal region to assess the mRNA expressions of uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma (PPARγ).
Results: We found no associations between the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs with REE, PA levels and diet ($p > 0.05$). However, the *PGC-1α*, *PPARα* and *PPARγ* mRNAs were more expressed in individuals displaying moderate rather than low PA levels ($p < 0.05$). Furthermore, *PGC-1α*, *PPARα* and *PPARγ* mRNAs were negatively correlated with fat mass percentage ($p < 0.05$). *PGC-1α* and *PPARα* mRNAs were also negatively correlated with BMI, while *PGC-1α* mRNA was inversely associated with waist-to-hip ratio ($p < 0.05$).
Conclusion: REE, PA levels and diet are not associated with browning formation indices of subcutaneous adipose tissue in healthy adult men.

Keywords: brown-like adipocytes, exercise, IPAQ, nutrition, uncoupling protein 1
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Introduction

Brown adipose tissue (BAT) in humans is characterized by increased mitochondrial content and an increased ability to uncouple cellular respiration via the action of uncoupling protein 1 (UCP1) in response to cold exposure [1]. This mechanism appears to be important, as UCP1-mediated thermogenesis may represent up to 30% of resting energy expenditure (REE) in adult humans [2]. Animal studies have shown that increased BAT activity reduces weight gain, improves insulin sensitivity, lowers free fatty acid levels in serum and reduces the risk for type 2 diabetes and other metabolic disorders [3], [4], [5], [6], [7]. Interestingly, some studies show that regular exercise training may increase BAT activity in animals [8], [9], [10], [11], [12], while evidence in humans

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suggests that participation in habitual physical activity (PA) is associated with BAT activity in supraclavicular and spinal areas [13]. Finally, a low protein diet [14], [15] and a high-fat diet [16], [17], [18], [19] in animals may increase BAT activity due to diet-induced thermogenesis that occurs via UCP1 activity [20].

White adipocytes also express UCP1, which indicates a brown adipose-like phenotype that may be been linked to obesity resistance in animals [3], [5], [21]. Several animal studies have also shown that exercise may initiate the conversion of subcutaneous white adipose tissue (WAT) into a brown adipose-like phenotype indicated by the presence of UCP1 and several other genes [22], [23], [24], [25]. For instance, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is expressed in WAT [26], and peroxisome proliferator-activated receptor gamma (PPARγ) can stimulate genes that are involved in the differentiation of brown fat cells [27], [28]. Furthermore, in vitro studies reported that *PPARγ* enhances *UCP1* mRNA in both BAT [29] and WAT [30].

It was also hypothesized that a physical exercise mechanism can increase REE, leading to body weight loss, through the induction of a brown adipose-like phenotype in WAT [31]. More specifically, PGC-1α upregulates the production of fibronectin type III domain-containing protein 5 (FNDC5) in skeletal muscle [31]. FNDC5 is then cleaved and releases a product called irisin into the bloodstream [31]. Irisin may bind to the surface of white adipocytes, changing their phenotype into brown-like adipocytes [31]. This hypothesis was confirmed by the presence of UCP1 within WAT that may occur, at least in part, by the action of peroxisome proliferator-activated receptor alpha (PPARα) [31], a transcription factor having a key role in the browning process of animal WAT [32]. Furthermore, regular exercise training increases *PPARγ* mRNA in the WAT of mice [33], [34]. However, evidence in humans showed that regular exercise has no effect on *UCP1* mRNA in WAT [35]. Given that humans display white adipocytes that may contain UCP1 [36] – the only known contributor to initiate BAT activity [1] – the presence of UCP1 in WAT indicates a brown adipose-like phenotype [37].

Nutrition may also play a role in the presence of UCP1 in WAT. For instance, a ketogenic diet (high-fat, adequate-protein and low-carbohydrate) may increase UCP1 expression in epididymal WAT of mice independently of PA levels [38]. Overall, the actual in vitro and in vivo evidence is inadequate or incomplete to support the browning formation of WAT in response to regular exercise and nutrition. Regarding exercise, this is mainly due to different types of animals and different exercise interventions used, while relevant data from human studies are very limited. Similarly, evidence from human studies to examine the effects of diet on the browning formation of WAT barely exists. Therefore, the aim of the current study was to examine the relationship of REE, PA participation and diet with browning formation markers of subcutaneous WAT in healthy men.

Materials and methods

Participants and procedure

The study conformed to the standards set by the Declaration of Helsinki and was approved by the Ethics Committee of the University of Thessaly (protocol 698/2013). The inclusion criteria were: healthy adult men, non-smokers, no chronic disease and/or being under medication treatment. Thirty-two healthy men [age (years): 36.06 ± 7.36, body mass index (BMI): 27.06 ± 4.62 (kg/m^2)] were recruited by advertisements in a local newspaper in Trikala, Thessaly, Greece; the data collection started in July 2013 and ended in June 2014. The characteristics of the participants are shown in Table 1.

Table 1: Characteristics of the participants.

	Mean ± standard deviation
Age (years)	36.06 ± 7.36
Waist-to-hip ratio	0.91 ± 0.07
Body mass index (kg/m^2)	27.60 ± 4.62
Fat mass %	28.32 ± 8.57
Fat-free mass (kg)	52.90 ± 5.02
MET-minute/week	914 ± 736

MET, metabolic equivalent.

To assess energy and nutrient intake of the participants, we retrieved diet recalls from two weekdays and one weekend day that were randomly selected within a week prior to the measurements. On the day of the measurements, the participants visited the laboratory between 07:00 and 09:00 am and completed the “usual week”

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short form of the International Physical Activity Questionnaire (IPAQ) that has been validated for healthy Greek adults [39]. Subsequently, participants underwent the following anthropometry measurements: body height was measured using a Seca (Seca, Hamburg, Germany) stadiometer, body weight was measured using a precision scale (KERN & Sohn GmbH, Version 5.3, Balingen, Germany), while waist-to-hip ratio (WHR) was assessed using a tape measure. Percent body fat and fat-free mass were measured via bioelectrical impedance using a body composition monitor (Fresenius Medical Care AG & Co., Bad Homburg, Hessen, Germany). Finally, the participants underwent an assessment of REE by indirect calorimetry that was followed by a subcutaneous fat biopsy.

Assessment of REE

Participants were instructed to reach the research laboratory in a vehicle on the day of the measurement. REE assessments were conducted between 07:00 to 09:00 am following a 12-h fast, while participants refrained from exercise, alcohol and passive smoking during the 72 h prior to the measurements [40], [41]. REE was measured using an automated gas analyzer (Vmax, CareFusion, NJ, Yorba Linda, CA, USA) to record respiratory variables every 20 s in a supine position for 30 min in a quiet room maintained at 22–24 °C. From the 30 min of the collected data, the first and last 5 min were removed [41]. The remaining 20 min of the collected data were averaged to obtain the final REE (kcal) value [41]. Respiratory gas measurements were extracted using the Weir equation [42] to convert VO_2 and VCO_2 values to REE (kcal) values.

Assessment of PA levels

IPAQ is self-reported and was completed by each participant at the laboratory. Detailed explanations were provided to the participants to ensure the clarity of the questionnaire, while an investigator was continuously available for further clarification. The obtained data were transformed into weekly metabolic equivalent (MET-min/week) based on low, moderate and vigorous PA intensity and number of days/week and min/day of their PA participation, following the IPAQ guidelines [43]. More specifically, the following equations were used: (a) Walking (low level) = 3.3 MET × min of walking × days of activity, (b) Moderate intensity level = 4 MET × min of walking × days of activity and (c) High (vigorous) intensity level = 8 MET × min of walking × days of activity. The total MET-min/week for each participant was calculated according to the equation: MET-min/week = Walking (MET × min × days) + Moderate (MET × min × days) + High [(vigorous) (MET × min × days)] [43].

The PA categories were grouped based on the number of days/week, min/day and number of MET-min/week of PA [43]. Specifically, the PA level of the participants that did not meet the criteria for “moderate” and/or “high” levels of PA was classified as “low”. The PA level of the participants who reported (a) 3 or more days of vigorous-intensity activity of at least 20 min/day, or (b) 5 or more days of moderate-intensity activity and/or walking of at least 30 min/day or (c) 5 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities with a minimum total PA of at least 600 MET-min/week was classified as “moderate” [43]. Finally, the PA level of the participants who reported (a) vigorous-intensity activity on at least 3 days/week with a minimum total PA of at least 1500 MET-min/week or (b) 7 days/week of any combination of low, moderate or vigorous intensity activities with a minimum total PA of at least 3000 MET-min/week was classified as “high” [43].

Subcutaneous fat biopsies

All biopsies were executed by an experienced surgeon following a previous methodology [44] based on a standardized non-diathermy method. The participants underwent a subcutaneous fat biopsy after at least a 12-h fast [45]. Each participant was positioned on a surgical bed in a supine position. The site of the incision was disinfected and 10 mL of 2% xylocaine (no adrenaline) was injected in the region of the incision for local anesthesia. An incision was made until the adipose tissue was revealed, that was approximately 3–5 cm to the left or right of the navel while the incision length was approximately 2–2.5 cm. Subsequently, the subcutaneous tissue was removed with operating scissors and when the adipose tissue became visible, nearly 500 mg of adipose tissue was captured and removed. The collected adipose tissue sample was immediately immersed in liquid nitrogen at −190 °C. For the final deposition, the samples were placed in Eppendorf tubes and they were deposited in a freezer at −80 °C until analysis.

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Gene expression analysis

The analysis of gene expression was done by an investigator who was blinded to the aim of the study. Total RNA was extracted from the adipose tissue biopsies using an RNeasy Lipid Tissue mini kit (QIAGEN, Milan, Italy) following the manufacturer's protocol. First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega, MD, USA). Quantitative real-time polymerase chain reaction (DNA Engine Opticon® 2 System – Bio-Rad Laboratories, Inc., CA, 94547, USA) for the UCP1, PGC-1α, PPARα and PPARγ genes was performed using a Sybr Green fluorophore (GoTaq® Hot Start Polymerase – Promega Corporation, MD, USA). The change in fluorescence at every cycle was monitored and a threshold cycle above the background for each reaction was calculated. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out in at least duplicate for every sample. 18S rRNA gene was constantly expressed under all experimental conditions and was then used as a reference gene for normalization [46].

Assessment of diet

The participants were contacted by telephone – at 10:00 pm, after their last meal – on three separate randomly selected days (two weekdays, one weekend day) during a 1-week period for the completion of a 3-day diet record. All food and beverages consumed on the day of contact were recorded. All diet records were analyzed by a trained investigator using the software Nutritionist Pro, Version 5.4.0 (Axxya Systems, Redmond, WA, USA). This software has been previously used for research purposes [47], [48]. For the analysis, a search was conducted in Nutritionist Pro for each food and beverage consumed. Details regarding the amount and preparation of each food and/or beverage were also included. Once all the relevant information regarding the food or beverages was entered the software, a corresponding list of macro and micronutrient content was provided and subsequently saved. This process was repeated for all food and beverages listed on the diet record. If a food or beverage was not found within the Nutritionist Pro database, the investigator manually entered the macro and micronutrient content of that food and saved it to the database for future use. The feedback provided by the software for each food or beverage used in this study included the total energy intake (kcal), total weight of food (g), protein (g), carbohydrate (g), total fat (g), sugar (g) and caffeine (mg).

Statistical analysis

Following previous methodology, we removed the mean values (i.e. outliers) of *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs that were at a distance of more than two standard deviations from the mean of the distribution [13], [49]. This analysis resulted to the removal of two data points of PGC-1α, one of PPARα and two of PPARγ, while UCP1 values displayed no outliers. Non-parametric tests were used throughout. We examined associations of the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs with REE, PA (MET-min/week), energy intake (kcal), total weight of food (g), protein (g), carbohydrate (g), total fat (g), sugar (g) and caffeine (mg) as well as age, BMI, WHR, fat mass percentage and fat free mass (kg), using the Spearman correlation coefficient. We used the Mann-Whitney U test to assess differences in the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs due to variation in PA levels (low/moderate) and the Kruskal-Wallis analysis of variance with the post hoc Mann-Whitney U test to assess differences in the BMI categories (normal/overweight/obese). All analyses were conducted with SPSS (version 22; SPSS Inc., Chicago, IL, USA) and a $p \leq 0.05$ level of significance.

Results

We found no associations of the mRNAs of the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* genes with REE and PA levels ($p > 0.05$). The Mann-Whitney U test showed that the *PGC-1α* ($z = -2.468$, $p = 0.01$), *PPARα* ($z = -2.093$, $p = 0.04$) and *PPARγ* ($z = -1.998$, $p = 0.05$) mRNAs were more expressed in individuals displaying moderate than low PA levels (Figure 1), while the IPAQ data analysis revealed that none of the participants displayed high PA levels. Finally, we found no associations of the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs with age, energy intake (kcal), total weight of food (g), protein (g), carbohydrate (g), total fat (g), sugar (g) and caffeine (mg).

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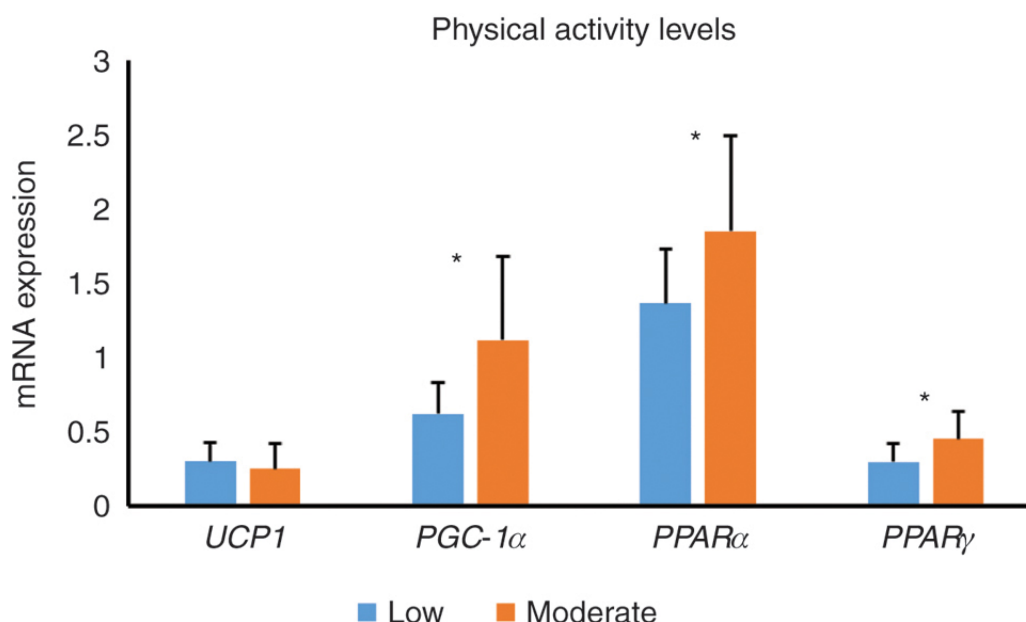

Figure 1: *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNA expressions of subcutaneous white adipocytes in healthy men due to variation of low and moderate physical activity levels.
*Significant differences between low and moderate levels of *PGC-1α* (p = 0.01), *PPARα* (p = 0.04) and *PPARγ* (p = 0.05) mRNA expressions. *UCP1*, uncoupling protein 1; *PGC-1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *PPARα*, peroxisome proliferator activated receptor alpha; *PPARγ*, peroxisome proliferator activated receptor gamma.

The *PGC-1α* mRNA was negatively correlated with fat mass percentage (rho = −0.69, p = 0.001) (Figure 2A), BMI (rho = −0.78, p = 0.001) (Figure 2B) and WHR (rho = −0.62, p = 0.001) (Figure 2C). Similarly, the *PPARα* mRNA was negatively associated with fat mass percentage (rho = −0.45, p = 0.01) (Figure 3A) and BMI (rho = −0.45, p = 0.01) (Figure 3B), while the *PPARγ* mRNA was negatively correlated with BMI (rho = −0.40, p = 0.03) (Figure 4). Additionally, individuals with normal BMI (19–24.9 kg/cm^2) revealed increased *PGC-1α* mRNA expression (z = −2.276, p = 0.02) compared to overweight individuals (BMI = 25–29.9 kg/m^2) (Figure 5). Also, individuals with normal BMI revealed increased *PGC-1α* mRNA (z = −3.220, p = 0.001), *PPARα* mRNA (z = −1.987, p = 0.05) and *PPARγ* mRNA expressions (z = −2.167, p = 0.03) compared to obese individuals (BMI ≥ 30 kg/m^2) (Figure 5). Finally, overweight individuals (BMI = 24.9–29.9 kg/cm^2) revealed increased *PGC-1α* mRNA expression (z = −2.325, p = 0.02) compared to obese individuals (BMI ≥ 30 kg/m^2) (Figure 5).

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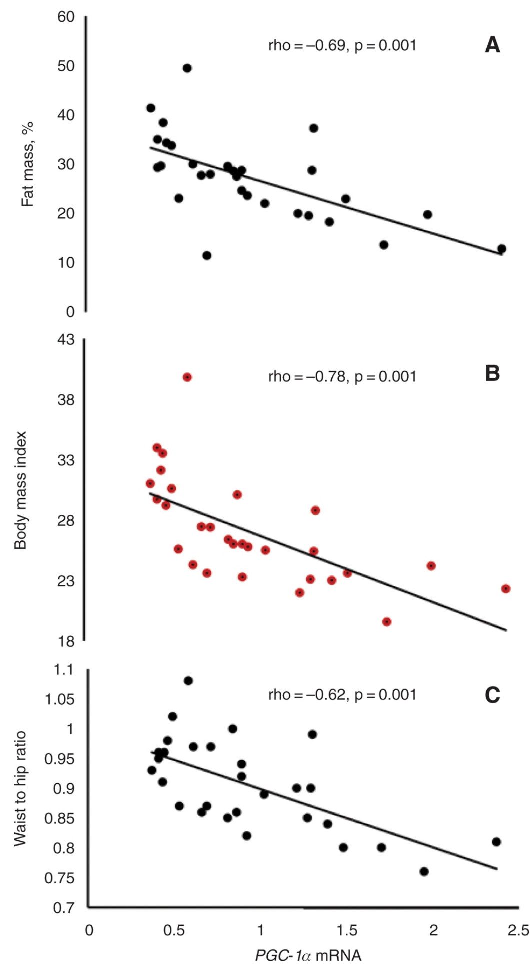

Figure 2: Associations of fat mass, body mass index and waist-to-hip ratio with *PGC-1α* mRNA expression of subcutaneous white adipocytes in healthy men.
PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

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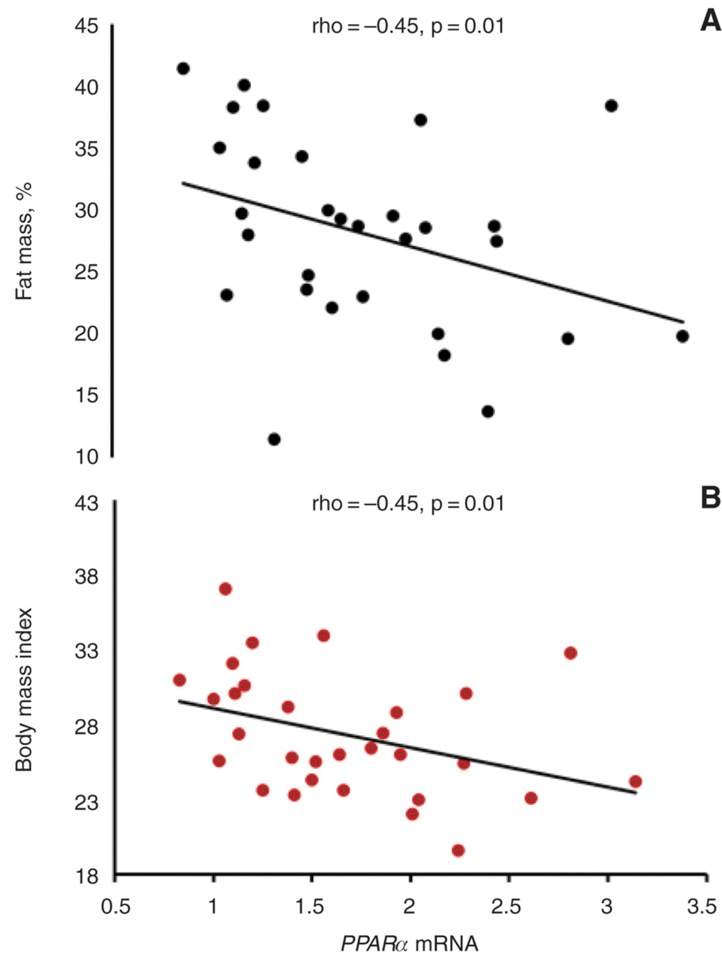

Figure 3: Associations of fat mass and body mass index with *PPARα* mRNA expression of subcutaneous white adipocytes in healthy men.
PPARα, peroxisome proliferator-activated receptor alpha.

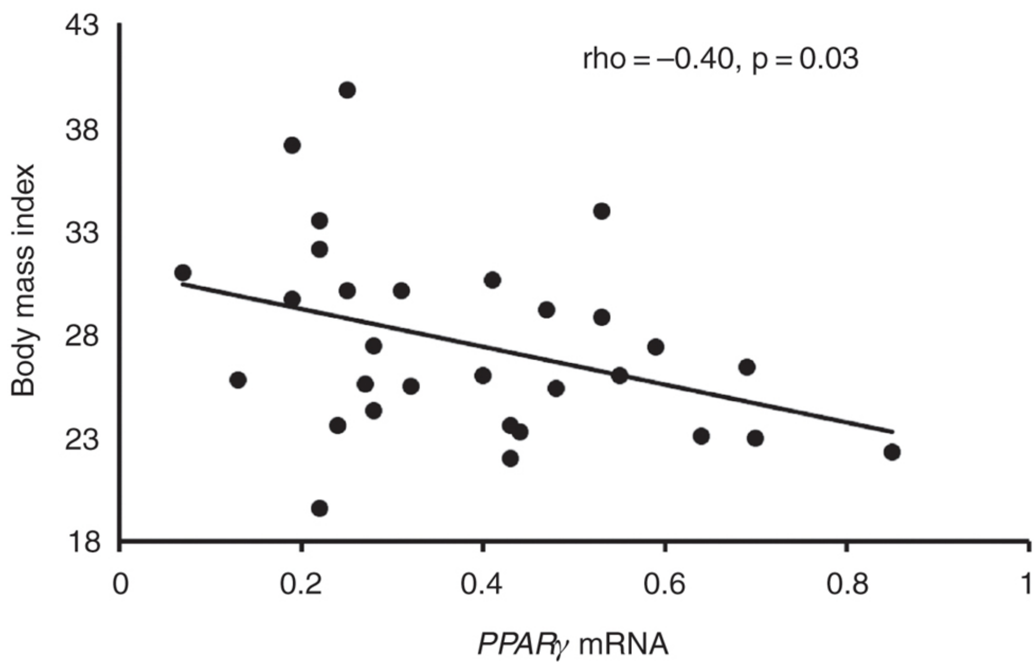

Figure 4: Associations of body mass index with *PPARγ* mRNA of subcutaneous white adipocytes in healthy men.
PPARγ, peroxisome proliferator-activated receptor gamma.

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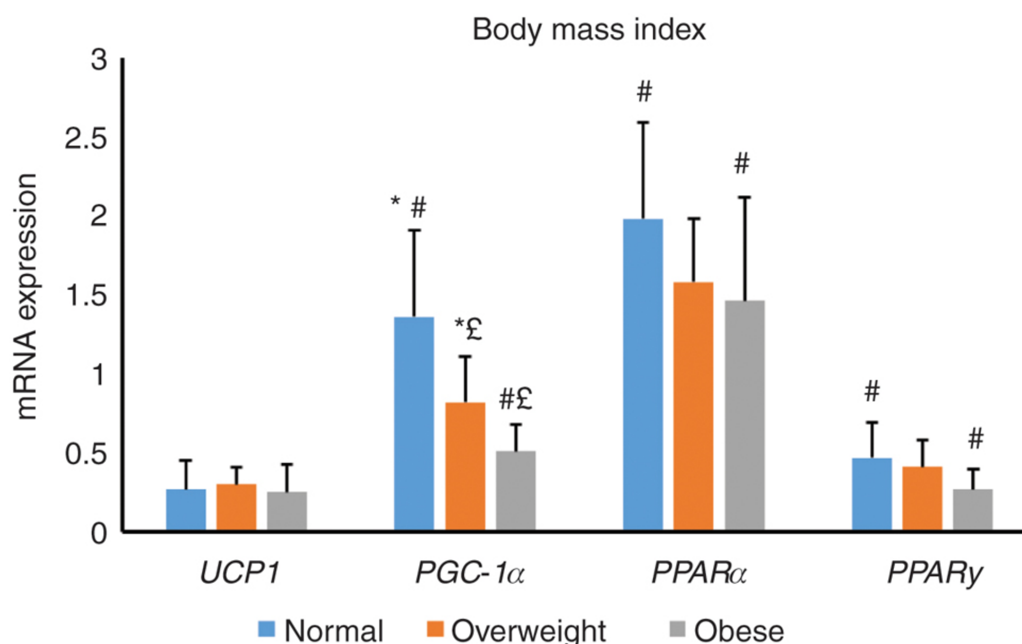

Figure 5: *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNA expressions of subcutaneous white adipocytes in healthy men due to variation of body mass index.
*Significant differences between normal and overweight individuals of *PGC-1α* mRNA (p = 0.02).
#Significant differences between normal and obese individuals of *PGC-1α* mRNA (p = 0.001), *PPARα* mRNA (p = 0.05) and *PPARγ* mRNA (p = 0.03). £, Significant differences between overweight and obese individuals of *PGC-1α* mRNA (p = 0.02). UCP1, uncoupling protein 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *PPARα*, peroxisome proliferator-activated receptor alpha; *PPARγ*, peroxisome proliferator-activated receptor gamma.

Discussion

The aim of the current study was to examine the relationship between REE, PA participation and diet with browning formation markers of subcutaneous WAT in healthy men. We found no associations between the *UCP1*, *PGC-1α*, *PPARα* and *PPARγ* mRNAs and REE. In contrast to previous evidence showing that UCP1 in WAT was positively correlated with REE and negatively with BMI and weight loss [31], the current study could not confirm any link between the *UCP1* mRNA in WAT and metabolism indices (i.e. REE, BMI, WHR, fat mass and fat-free mass). Considering the recent finding that the *UCP1* mRNA in WAT is negatively associated with weight loss in healthy women [50], we could hypothesize the opposite, i.e. increased REE and body weight loss could affect UCP1 in WAT. Nevertheless, this remains to be elucidated. Previous data also showed that PPARγ promotes a brown adipose-like phenotype of WAT [23], [30] and it would be expected to be positively associated with REE, as increased REE may be the result of browning formation in WAT [51], [52], [53]. However, the PPARγ activation in BAT may promote REE via fatty acid oxidation, although this process may be suppressed in WAT because of the repression of PPARγ by steroid receptor coactivator-2, which increases triglyceride accumulation and decreases free fatty acids in WAT [54]. This may explain, at least in part, the findings in the current study.

Based on a recent suggestion that regular exercise training may transform WAT cells into brown-like adipocytes [31], [55], we would expect to observe that individuals with increased PA levels would display increased *UCP1* mRNA – the direct marker of browning formation of WAT [37]. However, we found no association between *UCP1* mRNA and PA levels. This finding is in line with the only controlled trial published to date reporting a non-significant increase of the *UCP1* mRNA in the subcutaneous WAT of healthy adults in response to a 12-week exercise training intervention [35]. Furthermore, a recent single-group design study showed no change in the gene expression of browning markers (including *UCP1*) in the WAT of healthy women after a 16-week exercise training intervention [50].

As previously suggested, *PGC-1α*, *PPARα* and *PPARγ* mRNAs can affect *UCP1* to cause browning formation in WAT [30], [31], [56]. We found no associations of the mRNAs of the *PGC-1α*, *PPARα* and *PPARγ* genes with PA levels. However, we observed that individuals with moderate PA levels express more *PGC-1α*, *PPARα*, and *PPARγ* mRNAs than individuals with low PA levels. *PGC-1α* is increased in WAT in response to exercise in mice [23], [57]. In humans, however, *PGC-1α* mRNA did not change after an 8-week resistance training intervention [58]. PPARα was suggested to increase *UCP1* mRNA in WAT in response to exercise [31] while PPARγ of sub-

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cutaneous fat of mice was not altered in response to an 8-week exercise programme [59] or it was increased in response to chronic exercise in mice [33].

Collectively, activation of the *PGC-1α*, *PPARα* and *PPARγ* genes could indicate browning formation of WAT through the increase of *UCP1* expression [31], [32], [60]. Nevertheless, even though we observed that *PGC-1α*, *PPARα* and *PPARγ* mRNAs are more expressed in individuals that display moderate than low PA levels, this was not accompanied by a relevant association with *UCP1* mRNA. Also, we found no positive associations between *PGC-1α*, *PPARα*, and *PPARγ* mRNAs with REE. Increased REE may result from browning formation in WAT [51], [52], [53] while PA levels are positively associated with REE in humans [61]. Notably, we did not detect an association between PA levels and REE in the current study. Furthermore, we found that none of the genes examined in the current study are associated with the diet of our participants. In this regard, a previous animal study showed that a ketogenic diet (high-fat, adequate-protein and low-carbohydrate) could increase UCP1 in WAT [38]. Our participants did not follow a ketogenic diet, which may explain our findings.

The inverse association of *PGC-1α* mRNA with fat mass, BMI and WHR indicates a low demand of energy in WAT, given that *PGC-1α* increases fatty acid oxidation in mice [62], [63], which designates increased demands of energy. An inverse association between *PPARα* mRNA with fat mass and BMI was also found. *PPARα* is activated under conditions of energy deprivation [64] and its activation promotes the catabolism of fatty acid beta oxidation [65]. Given that WAT is mainly responsible for energy storage [66], the inverse associations of *PPARα* mRNA with fat mass and BMI indicate increased energy storage in WAT. Furthermore, *PPARγ* mRNA was inversely associated with BMI in the current study. *PPARγ* increases fatty acid uptake in WAT via the improvement of insulin sensitivity [67], while it increases glucose uptake in WAT [68]. Given that free fatty acids are positively correlated with increased adiposity in adults [69], the observed inverse association of *PPARγ* mRNA with BMI in the current study seems believable.

It is reasonable to assume that the present results may have been influenced by methodological limitations such as the lack of a prior power calculation to determine an appropriate sample size. However, this was not possible given the lack of similar design studies that could be utilised to perform sample size calculations. Therefore, a post-measurement power calculation was conducted using an online software (DSS Research) to test >95% statistical power. This revealed 100% of power based on the *UCP1* mRNA value (0.27 ± 0.15) we detected in our study and the expected *UCP1* mRNA value (0.00010 ± 0.0003) from a previous controlled trial that examined the effect of exercise on the *UCP1* mRNA of subcutaneous WAT in humans [35]. The study only measured mRNAs of the genes examined. The quantity of mRNA indicates the transcription levels of a specific gene that could be associated with protein concentrations. However, it is well known that protein concentrations cannot be predicted from mRNA levels [70], [71], [72], as one molecule of mRNA may be used several times to encode the same protein [70], [71], [72]. Therefore, an analysis of the protein concentrations should be performed in the future to assess whether these proteins are involved in the browning formation of WAT in humans.

UCP1 mRNA is stimulated by thyroid hormones such as triiodothyronine [73]. Unfortunately, we were unable to measure thyroid hormones in our study to determine any potential relationship with *UCP1* mRNA. Even though the thyroid hormones were not measured, our participants had no clinical symptoms or characteristics of overt hypothyroidism. Also, hypothyroidism is more common in women than in men (~7-fold) [74]. Given that our participants were only men, we are confident that their thyroid status could not affect *UCP1* mRNA. Additionally, it is important to note that our participants were not highly active, as we did not detect individuals with high PA levels. Furthermore, even though a 3-day diet recall is considered an optimal tool to assess diet [75], it may also underestimate the energy intake [76]. Finally, our findings are different from those in animal studies, which can be primarily explained by the different conditions that our participants were exposed to compared to animals (i.e. PA levels, diet).

Conclusion

We conclude that REE, PA levels and diet are not associated with browning formation markers of subcutaneous adipose tissue in healthy men. This reflects the conditions that our participants were exposed to regarding the PA levels and diet. *UCP1* mRNA in WAT is not linked to body weight and composition (i.e. BMI, WHR and fat mass). *PGC-1α*, *PPARα* and *PPARγ* mRNAs are negatively associated with increased fat mass accumulation parameters (BMI, WHR and fat mass), suggesting a positive association of *PGC-1α*, *PPARα* and *PPARγ* with lipid catabolism in WAT. The mechanisms of the latter associations remain to be determined.

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Author Statement

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Conflict of interest: Authors state no conflict of interest.

Informed consent: Informed consent has been obtained from all individuals included in the study.

Ethical approval: The research related to human use complied with all the relevant national regulations and institutional policies, was performed in accordance with the tenets of the Helsinki declaration and has been approved by the authors' institutional review board or equivalent committee.

References

[1] Mattson MP. Perspective: does brown fat protect against diseases of aging?. Ageing Res Rev. 2010;9:69–76.
[2] van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301:R285–96.
[3] Kopecky J, Clarke G, Enerback S, Spiegelman B, Kozak LP. Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J Clin Invest. 1995;96:2914–23.
[4] Kopecky J, Rossmeisl M, Hodny Z, Syrovy I, Horakova M, Kolarova P. Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. Am J Physiol. 1996;270(5 Pt 1):E776–86.
[5] Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P, Enerback S. FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. Cell. 2001;106:563–73.
[6] Tsukiyama-Kohara K, Poulin F, Kohara M, DeMaria CT, Cheng A, Wu Z, et al. Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. Nat Med. 2001;7:1128–132.
[7] Seale P, Kajimura S, Spiegelman BM. Transcriptional control of brown adipocyte development and physiological function – of mice and men. Genes Dev. 2009;23:788–97.
[8] Yamashita H, Yamamoto M, Sato Y, Izawa T, Komabayashi T, Saito D, et al. Effect of running training on uncoupling protein mRNA expression in rat brown adipose tissue. Int J Biometeorol. 1993;37:61–4.
[9] Ozden H, Ozbag D, Akyuz F, Sunal E, Gurer F, Inal M. The effect of melathonin on changes in brown and white adipose tissue ratios induced by exercise. The significance of age in this effect. Saudi Med J. 2004;25:1753–4.
[10] Schroeder M, Shbiro L, Gelber V, Weller A. Post-weaning voluntary exercise exerts long-term moderation of adiposity in males but not in females in an animal model of early-onset obesity. Horm Behav. 2010;57:496–505.
[11] Oh KS, Kim EY, Yoon M, Lee CM. Swim training improves leptin receptor deficiency-induced obesity and lipid disorder by activating uncoupling proteins. Exp Mol Med. 2007;39:385–94.
[12] Xu X, Ying Z, Cai M, Xu Z, Li Y, Jiang SY, et al. Exercise ameliorates high-fat diet-induced metabolic and vascular dysfunction, and increases adipocyte progenitor cell population in brown adipose tissue. Am J Physiol Regul Integr Comp Physiol. 2011;300:R1115–25.
[13] Dinas PC, Nikaki A, Jamurtas AZ, Prassopoulos V, Efthymiadou R, Koutedakis Y, et al. Association between habitual physical activity and brown adipose tissue activity in individuals undergoing PET-CT scan. Clin Endocrinol (Oxf). 2015;82:147–54.
[14] Rothwell NJ, Stock MJ, Tyzbir RS. Energy balance and mitochondrial function in liver and brown fat of rats fed "cafeteria" diets of varying protein content. J Nutr. 1982;112:1663–72.
[15] Rothwell NJ, Stock MJ, Tyzbir RS. Mechanisms of thermogenesis induced by low protein diets. Metabolism. 1983;32:257–61.
[16] Leblanc J. Prefeeding of high fat diet and resistance of rats to intense cold. Can J Biochem Physiol. 1957;35:25–30.
[17] Yoshimura M, Hori S, Yoshimura H. Effect of high-fat diet on thermal acclimation with special reference to thyroid activity. Jpn J Physiol. 1972;22:517–531.
[18] Kuroshima A, Doi K, Yahata T, Ohno T. Improved cold tolerance and its mechanism in cold-acclimated rats by high fat diet feeding. Can J Physiol Pharmacol. 1977;55:943–50.
[19] Mercer SW, Trayhurn P. Effect of high fat diets on the thermogenic activity of brown adipose tissue in cold-acclimated mice. J Nutr. 1984;114:1151–8.
[20] Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009;9:203–9.
[21] Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature. 2008;454:961–7.
[22] Stanford KI, Middelbeek RJ, Townsend KL, Lee MY, Takahashi H, So K, et al. A novel role for subcutaneous adipose tissue in exercise-induced improvements in glucose homeostasis. Diabetes. 2015;64:2002–14.
[23] Sutherland LN, Bomhof MR, Capozzi LC, Basaraba SA, Wright DC. Exercise and adrenaline increase PGC-1{alpha} mRNA expression in rat adipose tissue. J Physiol. 2009;587(Pt 7):1607–17.
[24] Trevellin E, Scorzeto M, Olivieri M, Granzotto M, Valerio A, Tedesco L, et al. Exercise training induces mitochondrial biogenesis and glucose uptake in subcutaneous adipose tissue through eNOS-dependent mechanisms. Diabetes. 2014;63:2800–11.
[25] Cao L, Choi EY, Liu X, Martin A, Wang C, Xu X, et al. White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis. Cell Metab. 2011;14:324–38.

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[26] Dillon LM, Rebelo AP, Moraes CT. The role of PGC-1 coactivators in aging skeletal muscle and heart. IUBMB Life. 2012;64:231–41.
[27] Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998;92:829–39.
[28] Puigserver P, Spiegelman B. Peroxisome proliferator-activated receptorgamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev. 2003;24:78–90.
[29] Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta. 2001;1504:82–106.
[30] Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J Biol Chem. 2010;285:7153–64.
[31] Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature. 2012;481:463–8.
[32] Rachid TL, Penna-de-Carvalho A, Bringhenti I, Aguila MB, Mandarim-de-Lacerda CA, Souza-Mello V. Fenofibrate (PPARalpha agonist) induces beige cell formation in subcutaneous white adipose tissue from diet-induced male obese mice. Mol Cell Endocrinol. 2015;402:86–94.
[33] Li M, Bai Y, Chen C, Cui J, Xu X, Dai Y. Effects of exercise and conjugated linoleic acid on PPARgamma in adolescent obese rats. Wei Sheng Yan Jiu. 2015;44:179–84.
[34] Tanaka G, Kato H, Izawa T. Endurance exercise training induces fat depot-specific differences in basal autophagic activity. Biochem Biophys Res Commun. 2015;466:512–7.
[35] Norheim F, Langleite TM, Hjorth M, Holen T, Kielland A, Stadheim HK, et al. The effects of acute and chronic exercise on PGC-1alpha, irisin and browning of subcutaneous adipose tissue in humans. FEBS J. 2014;281:739–49.
[36] Enerback S. Human brown adipose tissue. Cell Metab. 2010;11:248–52.
[37] Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown?. Genes Dev. 2013;27:234–50.
[38] Srivastava S, Kashiwaya Y, King MT, Baxa U, Tam J, Niu G, et al. Mitochondrial biogenesis and increased uncoupling protein 1 in brown adipose tissue of mice fed a ketone ester diet. FASEB J. 2012;26:2351–62.
[39] Papathanasiou G, Georgoudis G, Papandreou M, Spyropoulos P, Georgakopoulos D, Kalfakakou V, et al. Reliability measures of the short International Physical Activity Questionnaire (IPAQ) in Greek young adults. Hellenic J Cardiol. 2009;50:283–94.
[40] Jamurtas AZ, Koutedakis Y, Paschalis V, Tofas T, Yfanti C, Tsiokanos A, et al. The effects of a single bout of exercise on resting energy expenditure and respiratory exchange ratio. Eur J Appl Physiol. 2004;92:393–8.
[41] Adriaens MP, Schoffelen PF, Westerterp KR. Intra-individual variation of basal metabolic rate and the influence of daily habitual physical activity before testing. Br J Nutr. 2003;90:419–23.
[42] Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol. 1949;109:1–9.
[43] International Physical Activity Questionnaire. [http://www.ipaq.ki.se/]. Accessed: May 20, 2013.
[44] Mutch DM, Tordjman J, Pelloux V, Hanczar B, Henegar C, Poitou C, et al. Needle and surgical biopsy techniques differentially affect adipose tissue gene expression profiles. Am J Clin Nutr. 2009;89:51–7.
[45] Campbell KL, Makar KW, Kratz M, Foster-Schubert KE, McTiernan A, Ulrich CM. A pilot study of sampling subcutaneous adipose tissue to examine biomarkers of cancer risk. Cancer Prev Res (Phila). 2009;2:37–42.
[46] Nedergaard J, Cannon B. UCP1 mRNA does not produce heat. Biochim Biophys Acta. 2013;1831:943–9.
[47] Stuempfle KJ, Hoffman MD, Hew-Butler T. Association of gastrointestinal distress in ultramarathoners with race diet. Int J Sport Nutr Exerc Metab. 2013;23:103–9.
[48] Michaliszyn SF, Shaibi GQ, Quinn L, Fritschi C, Faulkner MS. Physical fitness, dietary intake, and metabolic control in adolescents with type 1 diabetes. Pediatr Diabetes. 2009;10:389–4.
[49] Ratcliff R. Methods for dealing with reaction time outliers. Psychol Bull. 1993;114:510–32.
[50] Nakhuda A, Josse AR, Gburcik V, Crossland H, Raymond F, Metairon S, et al. Biomarkers of browning of white adipose tissue and their regulation during exercise- and diet-induced weight loss. Am J Clin Nutr. 2016;104:557–65.
[51] Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nat Med. 2013;19:1252–63.
[52] Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. Nat Cell Biol. 2013;15:659–67.
[53] Wu MV, Bikopoulos G, Hung S, Ceddia RB. Thermogenic capacity is antagonistically regulated in classical brown and white subcutaneous fat depots by high fat diet and endurance training in rats: impact on whole-body energy expenditure. J Biolo Chem. 2014;289:34129–40.
[54] Koppen A, Kalkhoven E. Brown vs. white adipocytes: the PPARgamma coregulator story. FEBS Lett. 2010;584:3250–9.
[55] Castillo-Quan JI. From white to brown fat through the PGC-1alpha-dependent myokine irisin: implications for diabetes and obesity. Dis Model Mech. 2012;5:293–5.
[56] Rachid TL, Penna-de-Carvalho A, Bringhenti I, Aguila MB, Mandarim-de-Lacerda CA, Souza-Mello V. PPAR-α agonist elicits metabolically active brown adipocytes and weight loss in diet-induced obese mice. Cell Biochem Funct. 2015;33:249–56.
[57] Rocha-Rodrigues S, Rodriguez A, Gouveia AM, Goncalves IO, Becerril S, Ramirez B, et al. Effects of physical exercise on myokines expression and brown adipose-like phenotype modulation in rats fed a high-fat diet. Life Sci. 2016165;100–8.
[58] Alvehus M, Boman N, Soderlund K, Svensson MB, Buren J. Metabolic adaptations in skeletal muscle, adipose tissue, and whole-body oxidative capacity in response to resistance training. Eur J Appl Physiol. 2014;114:1463–71.
[59] Petridou A, Tsalouhidou S, Tsalis G, Schulz T, Michna H, Mougios V. Long-term exercise increases the DNA binding activity of peroxisome proliferator-activated receptor gamma in rat adipose tissue. Metabolism. 2007;56:1029–36.
[60] Fukui Y, Masui S, Osada S, Umesono K, Motojima K. A new thiazolidinedione, NC-2100, which is a weak PPAR-gamma activator, exhibits potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAy obese mice. Diabetes. 2000;49:759–67.
[61] WHO. Physical activity Fact sheet N°385, January 2015 edn. Geneva, 2015.

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[62] Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature. 2001;413:179–83.
[63] Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature. 2001;413:131–8.
[64] Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. J Clin Invest. 1999;103:1489–98.
[65] Kersten S. Integrated physiology and systems biology of PPARalpha. Mol Metab. 2014;3:354–71.
[66] Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab. 2004;89:2548–56.
[67] Ahmadian M, Duncan RE, Sul HS. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. Trends Endocrinol Metab. 2009;20:424–8.
[68] Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, et al. PPARgamma signaling and metabolism: the good, the bad and the future. Nat Med. 2013;19:557–66.
[69] Frohnert BI, Jacobs DR, Steinberger J, Moran A, Steffen LM, Sinaiko AR. Relation between serum free fatty acids and adiposity, insulin resistance, and cardiovascular risk factors from adolescence to adulthood. Diabetes. 2013;62:3163–9.
[70] Cooper S, Shedden K. Microarrays and the relationship of mRNA variation to protein variation during the cell cycle. J Theor Biol. 2007;249:574–81.
[71] Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 2003;4:117.
[72] Maier T, Guell M, Serrano L. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 2009;583:3966–73.
[73] Martinez de Mena R, Scanlan TS, Obregon MJ. The T3 receptor beta1 isoform regulates UCP1 and D2 deiodinase in rat brown adipocytes. Endocrinology. 2010;151:5074–83.
[74] Garber JR, Cobin RH, Gharib H, Hennessey JV, Klein I, Mechanick JI, et al. Clinical practice guidelines for hypothyroidism in adults: cosponsored by the American Association of Clinical Endocrinologists and the American Thyroid Association. Thyroid. 2012;22:1200–35.
[75] Ma Y, Olendzki BC, Pagoto SL, Hurley TG, Magner RP, Ockene IS, et al. Number of 24-hour diet recalls needed to estimate energy intake. Ann Epidemiol. 2009;19:553–9.
[76] Program IoMUCoDRAitW: Dietary Risk Assessment in the WIC Program. Washington (DC): National Academies Press (US). 5 Food-Based Assessment of Dietary Intake 2002.

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