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Whole-Body Lipid Oxidation during Exercise is Correlated to Insulin Sensitivity and Mitochondrial Function in Middle-Aged Obese Men

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Abstract
Defects in insulin sensitivity are associated with metabolic inflexibility and obesity albeit some obese subjects have preserved insulin sensitivity. A decrease in lipid oxidation during exercise can be found in obesity and type 2 diabetes. This lipid oxidation capacity has been found to be associated in healthy or overweight subjects with insulin sensitivity and mitochondrial function. Thus, we aimed at delineate the interrelationships among the maximal ability to oxidize lipids at exercise (LIPOXmax) insulin sensitivity and mitochondrial function in middle-aged obese men with different levels of insulin sensitivity.

After an overnight fast, twenty middle-aged male obese subjects performed: a maximal incremental exercise test allowing the determination of LIPOXmax, two days after a measurement of insulin sensitivity and two days after a muscle biopsy for mitochondrial respiration.

According to the minimal model, we found 9 insulin-resistant and 11 insulin-sensitive middle-aged obese men. LIPOXmax (expressed as power intensity) and whole-body insulin sensitivity are significantly correlated in obese insulin-sensitive but not in insulin-resistant obese men. LIPOXmax and insulin sensitivity are correlated to mitochondrial pyruvate oxidation and mitochondrial density. Multivariate analysis indicates that insulin sensitivity is statistically better explained by the LIPOXmax which is in turn better explained by the mitochondrial function parameter Vmax/V0 pyruvate.

Thus, insulin sensitivity, mitochondrial function and the ability of muscle to oxidize lipids during exercise are three closely correlated parameters in middle-aged male obese insulin-sensitive patients.

Keywords: Insulin sensitivity; LIPOXmax; Mitochondrial respiration

Introduction
Low insulin sensitivity is a common defect in obesity and type 2 diabetes [1] and is associated with a loss of metabolic flexibility [2] and, although this aspect is more controversial [3], with impaired mitochondrial function [4,5]. The report by several teams of a decrease in the ability to oxidize lipids at exercise [6-8], a parameter which is correlated to mitochondrial respiration in type 2 diabetic patients [9], is likely to be related to these pathophysiological mechanisms [10,11]. Moreover, inter-subject variation in maximal fat oxidation during exercise is quite large (49-93%) [12] and not fully explained although gender [13], training [9,14,15] or nutritional status [16] appear to be major determinants of this variability. Moreover, maximal fat oxidation has been shown to be correlated to the distribution of type I fibers [12], lean body mass and maximal oxygen consumption in overweight men [17], 24-hour fat oxidation [18] and surrogates of insulin sensitivity in young, healthy men [18]. Alteration in skeletal muscle lipid oxidation seems to be an early event in the development of Insulin Resistance (IR) [19] and leads to the accumulation of intramyocellular lipid intermediates [10] which in turn inhibits the insulin signaling pathway [20] and insulin sensitivity [21]. Moreover, reduced fat oxidation ability in resting condition has previously been reported to predict future body composition. Thus, high respiratory quotient implying low fat oxidation has been associated with a...
Materials and Methods

Ethical approval

Informed consent was obtained from all subjects after explanation about the nature of the study and the risks related to their participation to the experiments. The study was approved by our local Ethics Committee (# 03/10/GESE) and was registered as a clinical trial under the number NCT01644942.

Experimental design

Twenty middle-aged male obese subjects participated to this study. Subjects were sedentary, measured by the VOORRIPS index [25], and took no medications.

Each subject was asked to come three times to the laboratory at 8 AM after an overnight fast. The first time was for the enrolment visit, which included clinical examination, a physical activity questionnaire, anthropometric measurements and the signature of the informed consent. Weight and height were collected and Body Mass Index (BMI) was calculated as follows: weight in kilograms divided by height in meter squared (kg/m²). Two days later, the subjects came back to the laboratory to perform an exercise test (see below). After two days, measurement of insulin sensitivity and glucose effectiveness with the minimal model and skeletal muscle biopsy of the vastus lateralis were performed.

Exercise test

All subjects were asked to come and perform the test in the morning after an overnight fast (12 hours). The test consisted of six minute steady-state workloads theoretically set at 20, 30, 40, 50, and 60% of Pmax. However, these intensity levels can be modified during the test according to the evolution of the respiratory exchange ratio (RER=VCO2/VO2) in order to obtain values of RER below and above 0.9 which is the level of the Crossover Point (COP) defined below. The subjects performed the test on an electromagnetically braked cycle ergometer (Ergoline Bosch 500). Heart rate and electrocardiographic parameters were monitored continuously throughout the test by standard 12-lead procedures. Metabolic and ventilatory responses were assessed using a digital computer based breath to breath exercise analyzing system (Jaeger MS-CPX). Thus, we could measure VO2 and VCO2 (in ml/min). Lipid oxidation (Lipox) and carbohydrate utilization (Glucox) rates were calculated by indirect calorimetry from gas exchange measurements according to the non-protein respiratory quotient technique. This technique provided carbohydrate and lipid oxidation rates at different exercise intensities. Additionally, after smoothing the curves, we calculated the two parameters quantifying the balance between carbohydrates and lipids induced by increasing exercise intensity: the Maximal Lipid Oxidation Point (LIPOXmax) and the Crossover Point (COP).

The LIPOXmax is the exercise intensity at which lipid oxidation reaches its maximal level before decreasing while carbohydrate utilization further increases. It is calculated as previously reported after smoothing of the curve plotting lipid oxidation as a function of power. The maximal lipid oxidation rate at the LIPOXmax was expressed in mg/kg/min. The Crossover Point (COP) is the exercise intensity at which the proportion of carbohydrate used to provide energy becomes predominant over lipid oxidation [15]. Beyond this point, the subject is referred to as “glucodependent”. It was calculated as the exercise intensity where 70% of the substrates used to provide energy are carbohydrates and 30% are lipids [26].

Insulin sensitivity

A frequent-sampling intravenous glucose tolerance test was performed according to the protocol usually employed in our unit [27]. After 12-hours fast, at 09h00 a cannula was placed in the cephalic vein at the level of the cubital fossa for blood sampling. A glucose injection (0.5 g/kg, solution at 30%) was administered in the contralateral cephalic vein slowly over precisely 3 min. Blood samples were drawn twice before the glucose bolus and 1, 3, 4, 8, 10, 15, 19, 20, 22, 30, 41, 70, 90, and 180 min after glucose injection. Insulin (0.02 U/kg body weight, i.e. 1 or 2 U) was injected iv immediately after the 19 min sample. The 1 and 3 min samples were used for determination of the insulin early secretory phase. The other samples were necessary for minimal model calculations.

The minimal model analysis of the IVGTT was performed according to Bergman’s method [28,29] with the software TISPAG from University of Montpellier (Montpellier, France) [30], which uses a nonlinear least square estimation. ST-MINIMOD was calculated from the following equations:

\[ dG(t)/dt = (p1 \cdot (X(t)) \cdot G(t), p1 \cdot Gb); \]
\[ G(0) = Go; \]
\[ dX(t)/dt: p2 \cdot X(t) \cdot p3 \cdot (I(t) - I_b); \]
\[ X(0)=0, \]

where G(t) and I(t) are plasma glucose and insulin concentrations, X(t) is the insulin concentration in a compartment remote from plasma (insulin action), and p1 to p3 are model parameters. Go is the glucose concentration that would be obtained immediately after injection if there were instantaneous mixing in the extracellular fluid compartment. Gb and I_b are the basal values of glucose and insulin. Parameter p1 represents the fractional disappearance rate of glucose independent of any insulin response. p3 and p2 determine the kinetics of insulin transport into and out of, respectively, the remote insulin compartment where insulin action is expressed. SI is an index of the...
Table 1: Subjects characteristics and comparison of balance of substrates and exercise performance parameters for all subject and insulin-sensitive and insulin-resistant subjects.

<table>
<thead>
<tr>
<th></th>
<th>All subjects</th>
<th>Insulin-sensitive</th>
<th>Insulin-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.1 ± 1.6</td>
<td>51.2 ± 2.6</td>
<td>55.5 ± 1.6</td>
</tr>
<tr>
<td>VO2RIPPS index</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.8</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>97.2 ± 2.3</td>
<td>94.0 ± 2.7</td>
<td>101.1 ± 3.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.9 ± 0.6</td>
<td>31.9 ± 0.7</td>
<td>33.9 ± 1</td>
</tr>
<tr>
<td>Body fat mass (kg)</td>
<td>30.0 ± 1.6</td>
<td>29.0 ± 2.1</td>
<td>31.2 ± 2.5</td>
</tr>
<tr>
<td>Body fat-free mass (kg)</td>
<td>67.1 ± 1.2</td>
<td>64.9 ± 1.5</td>
<td>69.8 ± 1.5</td>
</tr>
<tr>
<td>Total water (liter)</td>
<td>50.9 ± 1.1</td>
<td>48.5 ± 1.3</td>
<td>54.0 ± 1.3</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>109.6 ± 1.7</td>
<td>106.3 ± 2.0</td>
<td>113.6 ± 2.3</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>106.6 ± 1.2</td>
<td>104.1 ± 1.5</td>
<td>109.6 ± 1.4</td>
</tr>
<tr>
<td>Waist/Hip ratio</td>
<td>1.03 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Fasting plasma insulin (µU/ml)</td>
<td>11.6 ± 1.4</td>
<td>8.9 ± 1.2</td>
<td>15.3 ± 2.3</td>
</tr>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>5.4 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>SI (min⁻¹/µU/ml)x10⁴</td>
<td>3.6 ± 0.5</td>
<td>5.5 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.9 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>Sg (%/min)</td>
<td>2.3 ± 0.2</td>
<td>2.8 ± 0.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Fasting plasma cholesterol (mM)</td>
<td>5.39 ± 0.34</td>
<td>5.52 ± 0.39</td>
<td>5.21 ± 0.63</td>
</tr>
<tr>
<td>Fasting plasma HDL-cholesterol (mM)</td>
<td>11.1 ± 0.6</td>
<td>11.6 ± 0.06</td>
<td>1.04 ± 0.11</td>
</tr>
<tr>
<td>Fasting plasma LDL-cholesterol (mM)</td>
<td>3.49 ± 0.23</td>
<td>3.40 ± 0.28</td>
<td>3.60 ± 0.39</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130.9 ± 2.3</td>
<td>125.5 ± 2.4</td>
<td>138.9 ± 2.3</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80.9 ± 1.4</td>
<td>81 ± 1.8</td>
<td>80.9 ± 2.3</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>2.7 ± 0.4</td>
<td>3.4 ± 0.6</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Vmax VO2 pyruvate</td>
<td>3.17 ± 0.50</td>
<td>4.03 ± 0.86</td>
<td>2.22 ± 0.23</td>
</tr>
<tr>
<td>CS (µmol/min/g tissue)</td>
<td>12.9 ± 1.52</td>
<td>15.32 ± 2.39</td>
<td>9.72 ± 0.95</td>
</tr>
<tr>
<td>VO2 max (m/min/kg)</td>
<td>23.2 ± 1.2</td>
<td>24.6 ± 1.8</td>
<td>21.6 ± 1.2</td>
</tr>
<tr>
<td>Max power (watt)</td>
<td>150.8 ± 7.6</td>
<td>160.9 ± 12.2</td>
<td>138.4 ± 6.6</td>
</tr>
<tr>
<td>LIPOXmax (watt)</td>
<td>53.1 ± 3.7</td>
<td>57.0 ± 5.7</td>
<td>48.8 ± 4.3</td>
</tr>
<tr>
<td>COP (watt)</td>
<td>54.4 ± 5.8</td>
<td>62.3 ± 7.8</td>
<td>45.6 ± 8.2</td>
</tr>
</tbody>
</table>

Significantly different from obese insulin-sensitive subjects, p<0.05.

BM: Body Mass Index; COP: Crossover Point; CS: Citrate Synthase; HDL: High Density Lipoprotein; HOMA-IR: Homeostasic Model Assessment Of Insulin Resistance; HS-CRP: Ultrasensitive-C Reactive Peptide; LDL: Light Density Lipoprotein; Lipoxmax: Maximal Lipid Oxidation During Exercise Test; Sg: Glucose Assimilation; SI-MINIMOD Or SI: Insulin Sensitivity Determined by Minimal Model; Vmax/V0: Maximal Mitochondrial Respiration with ADP Divided by Basal Respiration.

**Skeletal muscle biopsies**

Vastus lateralis muscle biopsies were taken according to the percutaneous Bergström technique after local anesthesia (xylocaine), as routinely used in our laboratory [10]. The muscle samples (200-300mg) were divided in two portions. One part was immediately frozen in liquid nitrogen and stored at –80°C for enzymatic determinations (i.e., citrate synthase: CS activity). The other portion was used for the in situ mitochondrial respiration studies and was immediately placed in an ice-cold relaxing solution containing (mM): EGTA-calcium buffer: 10 (free Ca²⁺ concentration 100 nM), Imidazole: 20, KH₂PO₄: 3, MgCl₂: 1, Taurine: 20, DTT: 0.5, MgATP: 5 and, PCR: 15.

**Mitochondrial respiration**

Respiratory parameters of the total mitochondrial population were analysed in situ on fresh skeletal muscle fibres as previously described [31]. Bundles of muscle fibres were manually isolated and saponin-skinned (50 µg/ml saponin for 30 min at 4°C). In order to completely remove saponin and free ATP, the fibers were then washed with continuous stirring in relaxing solution for 10 min (4°C) followed by 2 x 5min washes in relaxation solution at 4°C (same composition as the relaxing solution, except that MgATP and phosphocreatine were replaced by 3 mM phosphate and 2mM fatty acid-free bovine serum albumin). After washing, the fibers were stored on ice till used. Respiration rates were determined at 27°C with a Clark electrode (Strathkelvin Instruments, Glasgow, Scotland) in an oxygraphic cell with continuous stirring in relaxation solution. Respiration rates were recorded in the presence of pyruvate (10 mM)/malate (2 mM) and palmitoyl-L-carnitine (40 µM). For each sample, basal oxygen consumption without ADP (V₀) was first recorded, and then the ADP-Stimulated Maximal Respiration (Vmax) was determined in the presence of a saturating concentration of ADP (2 mM). At the end of each measurement, cytochrome c was added to investigate the outer mitochondrial membrane integrity [32]. After the following respiratory measurements, the fibre bundles were dried overnight, and weighed the next day. Respiration rates were expressed in micromoles of O₂ per minute per gram of dry weight.

**Citrate Synthase (CS) activity**

Frozen muscle biopsies were homogenized in 40 volumes of a solution (pH 8.2) containing (mM): Tris HCl: 50, EDTA: 1 and MgSO₄: 5 in a glass homogenizer, as described by Srere et al. The crude homogenates were then centrifuged for 10 min at 500g and supernatants assayed for and CS activities. CS activity was measured with 0.38mM oxaloacetate and 1.2 mM acetyl-CoA. Enzyme activity was monitored by recording the changes in absorbance at 412 nm over 2.5 minutes at 30°C.

**Blood analysis**

Fasting insulin was assessed by radioimmunoassay (kit Bi Insuline IRMA, Schering CIS bio international, Marcoule, France) and plasma glucose concentrations with an automate (AU2700, Olympus, Rungis, France).

**Data analysis**

All statistical analyses were performed using a commercial software package (SigmaStat). Data are presented as means (±SEM). Significance of differences among obese insulin sensitive and obese insulin resistant were determined by using Student t-test. Correlations were determined by Spearman analysis. Multivariate analysis was performed using stepwise regression analysis. Statistical significance was accepted at p<0.05.

**Results**

Comparison of anthropometric, metabolic parameters, whole body oxidative capacity and balance of substrate during exercise in obese insulin-sensitive and obese insulin-resistant subjects.
Lambert K and Brun JF

Obese insulin-resistant men. BMI (R = -0.498, p = 0.026) and systolic blood pressure (R = -0.664, p = 0.005). We found a negative correlation between SI-MINIMOD and mitochondrial respiration (Vmax/V0 pyruvate; R = 0.613, p = 0.008). Similarly, LIPOXmax is significantly correlated to muscle oxidative parameters such as citrate synthase activity (R = 0.538, p = 0.026) as well as VO2 max expressed in ml/min/kg (R = 0.750, p < 0.001).

SI-MINIMOD was also correlated with oxidative parameters such as citrate synthase activity (R = 0.480, p = 0.044, Figure 1C) and mitochondrial respiration (Vmax/V0 pyruvate; R = 0.613, p = 0.005). We found a negative correlation between SI-MINIMOD and BMI (R = -0.498, p = 0.026) and systolic blood pressure (R = -0.664, p = 0.005).

Metabolic and insulin sensitivity parameters are not correlated in obese insulin-resistant men.

Table 2: Simple linear correlation analyses between LIPOXmax and oxidative parameters, metabolic variables for insulin-sensitive (n=11) and insulin-resistant (n=9) middle-aged obese men.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Insulin-sensitive</th>
<th>Insulin-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI-minimal model</td>
<td>r 0.701</td>
<td>p 0.024</td>
</tr>
<tr>
<td>VO2 max (ml/min/kg)</td>
<td>r 0.628</td>
<td>p 0.003</td>
</tr>
<tr>
<td>Vmax/V0 pyruvate</td>
<td>r 0.632</td>
<td>p 0.068</td>
</tr>
<tr>
<td>CS (µmol/min/g tissue)</td>
<td>r 0.597</td>
<td>p 0.089</td>
</tr>
</tbody>
</table>

When subjects were separated according to their insulin sensitivity, we found again a strong correlation between LIPOXmax and SI-MINIMOD or VO2 max and closed to significance for mitochondrial parameters only in obese insulin-sensitive subjects whereas these relations are lost in obese insulin-resistant subjects, Table 2.

Multivariate analysis

A stepwise regression analysis of the determinants of insulin sensitivity selects among correlated parameters (LIPOXmax, Vmax/V0 pyruvate, CS) only LIPOXmax (R = 0.636, p = 0.008) and excludes mitochondrial function parameters Vmax/V0 and CS. A stepwise regression analysis of the determinants of LIPOXmax selects among correlated parameters (insulin sensitivity, Vmax/V0 pyruvate, CS) only LIPOXmax (R = 0.636, p = 0.008) and excludes insulin sensitivity and CS. These two multivariate analyses indicate that insulin sensitivity is statistically better explained by LIPOXmax which is in turn better explained by the mitochondrial function parameter Vmax/V0 pyruvate.

Discussion

This study evidences for the first time in middle-aged obese nondiabetic male subjects that insulin sensitivity measured with the minimal model is proportional to the ability to oxidize lipids during exercise, which is in turn statistically explained by the mitochondrial function parameter Vmax/V0 pyruvate.

All methods employed in this study are largely validated. The
The importance of lipid oxidative capacity for the maintenance of insulin sensitivity has been highlighted in several studies, particularly in the context of insulin resistance [7,35,36]. There has been a growing body of evidence suggesting that correlation between insulin sensitivity and LIPOXmax is regularly reported in middle-aged obese males. Waist and hip circumferences, blood pressure, anthropometric parameters such as fat-free mass, and the ability of muscle to oxidize lipids during exercise are two closely correlated parameters in middle-aged male obese patients. Whole body insulin sensitivity is related to mitochondrial function, i.e. citrate synthase activity and mitochondrial respiration, and the ability of muscle to oxidize lipids during exercise seems to be closely related to skeletal muscle mitochondrial function, which is in line with the fact that most individuals [45] and stable over years [38]. Most authors employ the procedure with 3 min steps [35] but longer steps have been reported to yield a better steady state of gas exchange and thus to avoid possible bias in the estimation of the lipid oxidation rate particularly in obese subjects [39,40].

Lipid oxidation during exercise reaches a peak at a low to moderate intensity around 40% VO2 max followed by a decrease when intensity increases. This level is called FATmax in papers using the method of Achten [35] and LIPOXmax in papers using 6 min steps and derivation of the equation of lipid oxidation in order to detect the point where this derivative is equal to zero and indicates the top of the bell-shaped curve [26]. This peak of lipid oxidation is both decreased and shifted to lower intensities in situations of low insulin sensitivity such as obesity and diabetes [7,41]. Low intensity training targeted at this level reverses this defect [9,42] and is an easy way to prescribe efficient intervention to obtain prolonged fat loss and to improve carbohydrate and lipid metabolism [38,42,43]. It has also been suggested that this parameter is a marker of “metabolic fitness” [17,44]. Interestingly, this level of exercise is below that of usual sports, but is spontaneously selected for prolonged activity by most individuals [45] and thus represents the type of exercise that patients will perform when asked to increase their volume of activity over the week.

Therefore our finding of a positive relationship in obese middle-aged male between a reliable measurement of insulin sensitivity and maximal fat oxidation during exercise is not unexpected, although it has not yet been reported in this population. It is consistent with the findings of recent papers [17,18] which evidenced a correlation between maximal fat oxidation during exercise and a surrogate measurement of insulin sensitivity (Quantitative Insulin Sensitivity Check Index, QUICKI) in young, healthy men or with insulin sensitivity measured by hyper insulin micisoglycemic clamp in papers using 6 min steps [35] but longer steps have been reported to yield a better steady state of gas exchange and thus to avoid possible bias in the estimation of the lipid oxidation rate particularly in obese subjects [39,40].

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Whole-body fatty acid oxidation has been related to skeletal muscle mitochondrial fatty acid oxidation capacity [12] in young healthy men during low-intensity exercise. Citrate synthase activity and mitochondrial respiration had been shown to be higher in trained type 2 diabetic subjects who had also higher whole-body lipid oxidation rate during exercise [9] compared to untrained state. In our sample of obese subject’s are representative of all the spectrum of insulin sensitivity, we extend these findings to obese nondiabetic sedentary patients with or without insulin resistance and show that these parameters are correlated to the ability to oxidize lipids during exercise and to insulin sensitivity. The correlation between LIPOXmax and VO2max underpins the relation between lipid oxidation and whole-body oxidative capacity. The multivariate analysis suggests that the ability to oxidize lipids during exercise is a determinant of insulin sensitivity, presumably because lipid oxidation may prevent lipid-induced insulin resistance [42] via lipid metabolites [46]. In addition, as previously shown in diabetic subjects, the ability to oxidize lipids during exercise seems to be closely related to skeletal muscle mitochondrial function, which is in line with the fact that most of lipid oxidation occurs in oxidative muscle. Thus, interventions aiming at increasing mitochondrial function are likely to also result into an increase in lipid oxidation and in turn an increase in insulin sensitivity. Furthermore, we showed that only two weeks at this level of maximal lipid oxidation are sufficient for improving insulin sensitivity in class II and III obese man [42].

**Conclusion**

In conclusion, we have shown that insulin sensitivity, mitochondrial function, i.e. citrate synthase activity and mitochondrial respiration, and the ability of muscle to oxidize lipids during exercise are three closely correlated parameters in middle-aged male obese insulin-sensitive patients. Whole body insulin sensitivity is related to the ability to oxidize lipids during exercise, which in turn could be explained by skeletal muscle mitochondrial function. This leads to think that a low ability to oxidize lipids at exercise is one of the aspects of the multifaceted syndrome of insulin resistance.

**Acknowledgements**

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**Author Contributions**

References


