An intensive lifestyle intervention reduces circulating oxidised low-density lipoprotein and increases human paraoxonase activity in obese subjects

Angelo Russo\textsuperscript{a}, Irene Pirisinu\textsuperscript{b}, Carmine Vacca\textsuperscript{b}, Elisa Reginato\textsuperscript{a}, Emilia Sbroma Tomaro\textsuperscript{a}, Roberto Pippi\textsuperscript{a}, Cristina Aiello\textsuperscript{a}, Vincenzo Nicola Talesa\textsuperscript{b}, Pierpaolo De Feo\textsuperscript{a}, Rita Romani\textsuperscript{b,*}

\textsuperscript{a} Healthy Lifestyle Institute CURIAMO, Department of Medicine, University of Perugia, Polo Didattico Sant’Andrea delle Fratte, Piazzale Gambuli, 06132 Perugia, Italy
\textsuperscript{b} Department of Experimental Medicine, University of Perugia, Polo Didattico Sant’Andrea delle Fratte, Piazzale Gambuli, 06132 Perugia, Italy

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KEYWORDS
Exercise; oxLDL; PON activity; Obesity; Overweight.

Summary
Objective: Obesity has a great impact on cardiovascular morbidity and mortality, the treatment of this pathological state is important given the significant health consequences. Lifestyle and behaviour changes play a significant role in weight management. The purpose of this study was to investigate the impact of an intensive multidisciplinary lifestyle intervention on well-known atherogenic factors in a group of overweight and obese subjects.

Methods: A total of 44 people with overweight/obesity underwent a lifestyle intervention based on nutritional education, psychological support and a 3-month exercise training program with a frequency of twice a week. Several anthropometric and biochemical parameters were measured before and after the lifestyle intervention.
Introduction

Obesity is now a global epidemic and a major risk factor for chronic diseases that can accelerate morbidity and mortality. Scientific guidelines recommend lifestyle change and regular physical activity as primary therapeutic tools for people with obesity [1]. Regular physical exercise in population with obesity is associated not just with weight loss, but also significant improvement in the cardiovascular risk factors [1]. Some recent cross-sectional and longitudinal studies have demonstrated a significant positive association between circulating levels of oxidised low-density lipoprotein (oxLDL) and the body mass index (BMI), waist circumference, obesity, weight loss, and physical fitness [2].

Obesity is a multifactorial disease that appears to be influenced by both genetic and environmental factors [3]. In recent years, evidence has emerged showing that oxidative damage of lipoproteins may be linked with obesity [4,5]. Altered oxidant/antioxidant status and higher susceptibility LDL to lipid peroxidation have been reported in obese patients [4—7]. Increased adipose tissue volume promotes the development of glucose metabolism disturbances and insulin resistance, the factors known to strongly correlate with the accelerated progression of atherosclerosis [8].

The paraoxonase (PON) enzyme family consists of 3 members, namely PON1, PON2 and PON3 that share common chromosome location, structural and calcium-dependent ester hydrolase activities tightly associated with apoA-1 in HDL [9—11]. Although, the exact physiological role of PON is still unclear, they have the potential to prevent and reverse LDL oxidation [9—11]. It is thought that the presence of PON on HDLs protects LDL from lipid peroxidation [12]. Mice lacking PON1 activity appear to be highly susceptible to atherosclerosis [13,14]. ApoA-1 is the major apo in HDL particles and initiates the ‘reverse cholesterol transport’. ApoA-1 can ‘pick up’ excess cholesterol from peripheral cells and transfer it back to the liver in the HDL particles. ApoA-1 also manifests anti-inflammatory and antioxidant effects [15].

The aim of this study was to investigate the impact of a multidisciplinary lifestyle intervention on a series of parameters that increase cardiovascular risk including BMI, waist circumference, blood pressure, plasma glucose, total cholesterol, LDL, HDL, APO A1 and plasma triglycerides. Our working hypothesis was that in obese subjects, lifestyle intervention potentially increases PON activity which in turn influences the circulating levels of oxLDL.

Results: Lifestyle intervention led to a significant reduction in metabolic profile including body mass index (BMI), waist circumference, systolic and diastolic blood pressure, plasma glucose, and plasma triglycerides. These reductions were also accompanied by a significant increase in maximal oxygen consumption and muscle strength. Furthermore, paraoxonase and lactonase activities and the concentration of Apolipoproteins A1 (APO A1) were significantly increased and the serum levels of oxLDL reduced without any changes in the circulating levels of LDL and HDL.

Conclusion: In conclusion, our study suggests that an intensive lifestyle intervention in obese subjects promotes a series of beneficial antiatherogenic changes which included increased enzyme activities of paraoxonase and lactonase, concentration of Apolipoproteins A1 and decreased circulating levels of oxLDL.

Subjects

The participants were a subgroup of a multidisciplinary lifestyle intervention program at the Lifestyle Institute of the University of Perugia (CURIAMO). The CURIAMO trial (registered in the Australian New Zealand Clinical Trials Registry, ACTRN12611000255987) has been approved by the local Ethics Committee (CEAS Umbria Region, HREC number 1/10/1633). Between January 2012 and June 2014, 24 males and 20 females with a mean age of 54 ± 2 years (mean ± SE), affected by overweight (n = 13, BMI 28.4 ± 0.3) or obesity (n = 31, BMI 41.5 ± 4.2) were enrolled. Overweight and obesity were defined using the national BMI reference tables for age and sex. Among the
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<table>
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<tr>
<th></th>
<th><strong>U/mg</strong></th>
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<td>CuSO₄</td>
<td>T₀</td>
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<td>T₁</td>
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<td>Q2</td>
<td>Paraoxon activity</td>
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<td>T₁</td>
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<td>Q6</td>
<td>Paraoxon activity</td>
<td>T₀</td>
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Fig. 2 Specific activity of serum PON of obese subjects before and after physical activity and PON specific activity related to Q192R polymorphism. (A) PON activity with paraoxon substrate. (B) lactonase activity with DHC substrate. (C) PON1 and PON3 specific lactonase activity; to investigate whether the lactonase activity variation was attributable to PON1 or PON3 we performed assays in the presence and absence of CuSO₄ (inhibitor of PON3) or Co(NO₃)₂ (inhibitor of PON1). (D–E) PON1 activity variation in serum of obese subject after physical exercise related to Q192R. (D) Paraoxonase activity of PON1, (E) lactonase activity of PON1. The assay were performed in quadruplicate for both substrate; all the samples were incubated at room temperature for 2 h with 2 mM PMSF before the assay to inhibit carboxylesterase. To: the day before starting the intensive lifestyle intervention; T1: 3-months on the last day of the intensive lifestyle intervention. Data represent median (lower quartile-upper quartile). Statistical analysis was performed using Student’s t test. Asterisks indicate statistical significance **P < 0.01.

Materials and methods

Lifestyle intervention program

All subjects participated to the intensive phase of the lifestyle intervention program. The duration of this phase was of 3-months and characterised by an individualised exercise intervention as previously described in detail [16]. Briefly, during the intervention, patients underwent: (1) an initial medical examination; (2) an interview by a psychologist; (3) an assessment by a dietician; (4) a physical examination by a specialist in sports medicine; (5) an individualised program (groups of 5–6 patients) of 26 sessions (two per week) of structured indoor exercise; (6) 8 sessions of group therapeutic education, conducted by a doctor of pedagogical sciences. The exercise training program was performed in the gym and supervised by an exercise physiologist with a maximum attendance of 5 patient/group, twice a week for 3-months. The exercise intervention was a mixed aerobic and endurance training. Each of 26 sessions of 90 min was divided into 60 min of aerobic workout and 30 min of circuit training for muscular strength and flexibility exercises. The workout for muscular strength used isotonic machines for training of the lower and upper limbs, with gradual increase from 50% of 1 repetition maximum (RM). Aerobic capacity was estimated using the Rockport Fitness Test [17] on treadmill (Run race Technogym, Cesena, Italy). Determination of the maximum dynamic force of extensor muscles of the leg and the flexor and extensor muscles of the arms was conducted by Brzycki 1-RM prediction equation using leg press, leg extension, lat machine, and chest press machine (Technogym, Cesena, Italy) [18].

The aerobic workout was performed using ergometers for cardiovascular work (treadmill, cycle ergometer and...
Fig. 3 Levels of Apo A1 in CURIAMO patients before and after a multidisciplinary lifestyle intervention. To: the day before starting the intensive lifestyle intervention; T1:3- months on the last day of the intensive lifestyle intervention. Data represent median ± lower quartile-upper quartile, Statistical analysis was performed using Student’s t test. Asterisks indicate statistical significance **P < 0.01.

arm ergometer) increasing gradually the intensity of work from 50% heart rate reserve (HRR’s training range increasing 5% every three weeks) up to 70% HHR. The workout for muscular strength used isotonic machines for training of the lower and upper limbs, with a load corresponding to 50% of 1RM at the beginning, and two sets of 20 repetitions for each exercise and 45 s rest between sets and exercises. Gradually, the load was increased by 2.5/5 kg every three weeks.

**Blood sampling**

Venous blood samples were collected from all subjects in the morning after overnight fasting the day before starting the intensive lifestyle intervention and after 3 months on the last day of the intensive lifestyle intervention. Blood was transferred either in EDTA tubes (to conserve the whole blood for DNA extraction) or in serum tube (measurement of PON activity and levels of oxLDL). The samples were centrifuged for 10 min at 1000 x g at room temperature, and the supernatants stored at −20 °C for further analysis.

**Materials**

Tris[(hydroxymethyl)aminomethane], sodium chloride(NaCl), magnesium chloride (MgCl₂), calcium chloride (CaCl₂), Phenylmethanesulfonyl fluoride (PMSF), Dihydrocoumarin (DHC) paraoxon (diethyl-p-nitrophenyl phosphate), Cobalt nitrate Co(NO₃)₂, Copper(II) sulphate (CuSO₄), Bovine serum albumin (BSA) were purchased from Sigma–Aldrich S.r.l. (Milano, Italy) unless otherwise indicated. All solutions were made from Sigma—Aldrich S.r.l. (Milano, Italy) unless otherwise indicated. All solutions were made from twice-distilled water.

**Assay methods**

Protein concentrations were measured according to Lowry et al. [19] using BSA as a standard. Serum paraoxonase activity was determined according to Romani et al. [20]. The samples were incubated for 2 h at room temperature with 2 mM PMSF before the assay, in order to inhibit carboxylesterase. In order to distinguish between PON1 and PON3, we used substrates paraoxon and DHC. Indeed, PON1 has PON activity while its lactonase activity is restricted to lipophylic lactones; PON3 has no paraoxonase activity, very low arylesterase activity, and high lactonase activity. To distinguish the lactonase activity of PON1 and PON3, serum samples were incubated for 15 min at 37 °C with 0.1 mM Co(NO₃)₂, which inhibits PON1 (IC 50 = 0.08 mM), or with 0.06 mM CuSO₄, which inhibits PON3 (IC 50 = 0.036 mM) [21]. Nine subjects participated in the CURIAMO protocols were not included in the determination of PON activity because their therapy included statins and/or aspirin because these drugs might affect the enzymatic activity of PON [22].

Genomic DNA was extracted from EDTA tubes containing peripheral whole blood, using the DNeasy protocols (Life Technologies, Monza, Italy). Analysis of PON1-192 Q/R polymorphism was performed by PCR-restriction fragment length polymorphism according to Humbert et al. [23]. The levels of serum ox-LDL were measured using oxLDL/MDA Adduct sandwich ELISA Kit (Immundiagnostic AG, Bensheim, Germany) according to manufacturer’s instructions. The quantification of human Apolipoprotein A1 (APO A1) was performed by ELISA using specific kits (Apolipoprotein A1, ABCAM, Cambridge, UK).

**Statistical analysis**

Data were analysed using the SPSS 11.5 statistical package (SPSS Inc., Chicago, IL, United States). Statistical analysis was performed using Student’s t test for comparison between paired groups. Asterisks indicate statistical significance: *P < 0.05; **P < 0.01. Data are presented median (lower quartile-upper quartile). Relationships between parameters were assessed by Pearson correlation analysis performed with GraphPad program.

**Results**

At the end of 3-month of multidisciplinary lifestyle intervention program, obese subjects showed a significant decrease in BMI, waist circumference, systolic and diastolic blood pressure, plasma glucose, and plasma triglycerides. At the same time, maximal oxygen consumption and muscle strength were found to be significantly increased (Table 1). Specifically, the treatment period did not alter the levels of LDL, HDL and total cholesterol (Table 1), but significantly reduced the circulating oxLDL levels by 21% in all the patients (Fig. 1) Since, HDL-associated PONs are able to hydrolyse oxidised phospholipids and protect LDL from oxidative modifications, we evaluated the effect of multidisciplinary program on the PONs activity and a possible correlation with circulating levels of oxLDL. The PON activity was found to be increased by 37% (Fig. 2A) and lactonase activity by 26% (Fig. 2B). Since lactonase activity is determined by both PON1 and PON3, we performed the assay in the presence or absence of CuSO₄ (inhibitor of PON3) or Co (NO₃)₂ (inhibitor of PON1) in order to distinguish which of the two enzyme was responsible for the activity variation. We found that the contribution of PON1 and PON3 was the same; the activity of both was significantly increased.

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**Table 1** BMI, waist circumference, blood pressure, plasma glucose, total cholesterol, LDL, HDL cholesterol, plasma triglycerides, maximal oxygen consumption, and muscle strength before and after 3-month exercise training program in 44 overweight/obese subjects (values median (lower quartile-upper quartile), statistical analysis was performed using Student’s t test).

<table>
<thead>
<tr>
<th>N=44 subjects</th>
<th>Baseline</th>
<th>Intervention</th>
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<tbody>
<tr>
<td>Body mass index (kg/m²)</td>
<td>33.35 (29.30—39.50)</td>
<td>32.10 (28.15—36.60)*</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>104 (100—118)</td>
<td>100 (94—112)*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 (120—138.75)</td>
<td>115 (110—127.5)*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 (71.25—85)</td>
<td>70 (60—75)*</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>5.78 (4.81—7.08)</td>
<td>5.39 (4.64—6.78)*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>10.67 (9.57—12.54)</td>
<td>10.81 (9.44—11.85)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>2.67 (2.22—2.97)</td>
<td>2.61 (2.22—3)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>6.33 (5.33—7.78)</td>
<td>6.86 (5.28—7.74)</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>7.61 (5.49—9.74)</td>
<td>5.89 (4.44—8.72)*</td>
</tr>
<tr>
<td>VO2max (ml/kg/min)</td>
<td>19.10 (13.10—29.95)</td>
<td>30 (24.90—33.70)*</td>
</tr>
<tr>
<td>Muscle strength lat machine (kg)</td>
<td>41.70 (31.90—43.50)</td>
<td>49.70 (40.27—55.52)*</td>
</tr>
<tr>
<td>Muscle strength chest press (kg)</td>
<td>32.40 (24.80—42.15)</td>
<td>41.80 (31—52.52)*</td>
</tr>
<tr>
<td>Muscle strength leg press (kg)</td>
<td>155.45 (129.35—186.85)</td>
<td>216.10 (186.20—248.30)*</td>
</tr>
<tr>
<td>Muscle strength leg extension (kg)</td>
<td>29.40 (25.20—39.90)</td>
<td>49.85 (41.60—65.95)*</td>
</tr>
</tbody>
</table>

Asterisks indicate statistical significance *P < 0.01.

Discussion

Obesity, which is increasing at an alarming rate due to unhealthy lifestyles, is associated with a wide range of chronic health risks later in life, such as cancer, cardiovascular disease, and type 2 diabetes [24,25]. The correction of unhealthy lifestyles is possible through cost-effective measures and might prevent about 80% of cases of heart disease, stroke, and type 2 diabetes, and 40% of cancers [26]. To be successful, lifestyle interventions must take into account the perception of the intervention and the relationship with food and exercise of obese persons. At present, there is a need for effective and reproducible multidisciplinary models for the treatment of obesity using lifestyle changes [27]. The results of the present study demonstrate for the first time that an intensive lifestyle intervention decreases circulating oxLDL, increases the activity of antiatherogenic enzymes PON and the protein APO A1s. Moreover, our study confirms some previous findings showing positive effects of intensive lifestyle intervention on BMI,

by 14%. (Fig. 2C). The improvement of PON/oxLDL in the overweight and obese subjects was not statistically different.

In addition, we considered the genotype of all patients for polymorphic variant PON1 Q192R that may influence the capacity of enzyme to prevent oxidation of LDL. The results showed that 19 subjects were QQ, 24 individuals QR, and only one individual was RR. The significant changes of paraxonazone and lactonase activity (related to PON1) (Fig. 2D, E) were higher in subject QQ (44% and 28% respectively) than QR (26% and 15% respectively) as well as the decrease of oxLDL. Moreover, the levels of Apo A1 in the serum of patients before and after the lifestyle intervention, significantly increased (Fig. 3). A key finding of the study was the significant correlation between oxLDL vs. PON activity and oxLDL vs.VO2max, r = 0.5493, p = 0.0030 and r = 0.44, p = 0.0087, respectively (Fig. 4), in the absence of significant correlations between the variation of APO A1 and the levels of oxLDL or PON activities.

![Fig. 4](http://dx.doi.org/10.1016/j.orcp.2016.11.006)
waist circumference, blood pressure, plasma glucose and plasma triglycerides in obese subjects. To date, the precise effects of physical exercise on oxLDL and PON activities in humans have not been established. Some of the existing studies show contradictory findings especially because the type of physical exercise and the choice of subjects were different and often not comparable. Some studies have reported an improvement in LDL oxidation rate and an increase in the resistance of LDL to oxidation in young subjects following moderate intensity exercise training [28,29]. Different results were found in sedentary healthy young volunteers where regular exercise was not associated with an increase in the basal level of PON1 activity [30]. In their study, Otocka-Kmiecik et al. [31] found that PON1 activities at baseline were not significantly different between sedentary and sport athletes, but appeared to increase at the bout of maximal exercise only in physically active subjects. Koncos et al. show that modifications in dietary habits and physical activity might exert antiatherogenic changes in childhood obesity because significantly increase PON1 paraoxonase activity [8].

Our results show a positive effect of the lifestyle intervention on PON activities in overweight and obese subjects which should be clinically relevant since antiatherogenic PON activity is significantly lower in subjects with obesity in comparison to healthy controls [4,32]. In view of the above literature, our results further support the role of exercise and healthy nutrition for treatment of obesity and the reduction of the cardiovascular risk. In fact, since individuals with obesity have low levels of serum PON activity and less ability to prevent the oxidation of LDL, the beneficial effect of moderate exercise has important clinical implications. The moderate exercise, without pharmacological treatment, exerts beneficial effects on lipids via PON activity and thus demonstrates a novel antiatherogenic mechanism of exercise induced by exercise in obesity.

In conclusion, our study suggests that positive modifications in dietary habits and physical activity induce antiatherogenic changes and are potentially able to reduce the cardiovascular risk in obese patients, without specific pharmacological treatments. This study also shows a new cardioprotective mechanism exerted by lifestyle intervention in obese subjects which increases PONS activities and reduces the levels of oxLDL.

Authors contributions

RR, PDF, VNT conceived the experiments and in writing the paper. RR, AR, ES, RP, ER, IP, carried out the experiments and analyzed the data.

Acknowledgements

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References

[1] Klein S, Burke LE, Bray GA, Blair S, Allison DB, Pi-Sunyer Furlong CE. Genetic variability in the cytochrome P450-

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