Effects of an acute exercise on circulating extracellular vesicles: tissue-, sex- and BMI-related differences

Antonello E. Rigamonti[1], Valentina Bollati[2], Laura Pergoli[2], Simona Iodice[2], Alessandra De Col[3], Sofia Tamini[3], Sabrina Cicolini[3], Gabriella Tringali[3], Roberta De Micheli[3], Silvano G. Cella[1], Alessandro Sartorio[3]

1. University of Milan, Department of Clinical Sciences and Community Health, Milan, Italy

2. EPIGET LAB, University of Milan, Department of Clinical Sciences and Community Health, Milan, Italy

3. Istituto Auxologico Italiano, Experimental Laboratory for Auxo-endocrinological Research, Verbania and Milan, Italy

Running title: Post-exercise EVs in obese vs. lean subjects

Key words: extracellular vesicles, exercise, obese/lean, male/female, platelets, muscle, cell origin, HOMA-IR

Correspondence to: Dr. Antonello E. Rigamonti, MD, PhD - University of Milan, Department of Clinical Sciences and Community Health, via Vanvitelli 32, 20129 Milan, Italy - Tel.: +39 02 503 17013; FAX: +39 02 503 17011; email: antonello.rigamonti@unimi.it

24 **Abbreviations**

25 BMI, body mass index; bpm, beats per minute; CI, confidence interval; hCRP, human C-
26 reactive protein; CV, coefficient of variation; DBP, diastolic blood pressure; ED, exercise
27 duration; EVs, extracellular vesicles; F, female; FABP, fatty acid binding protein; FFM, fat-
28 free mass; FM, fat mass; HbA_{1C}, glycated hemoglobin; HDL-C, high-density lipoprotein
29 cholesterol; hip C, hip circumference; HOMA-IR, homeostatic model assessment of insulin
30 resistance; HR, heart rate; IQR, interquartile range; LCI, lower 95% confidence interval;
31 LDL-C, low-density lipoprotein cholesterol; M, male; NTA, nanoparticle tracking analysis;
32 NW, normal-weight; OB, obese; PM, particulate material; Ref, reference category used for
33 comparing pairs of times; SBP, systolic blood pressure; sd, standard deviation; se, standard
34 error; SCGA, sarcoglycan α; T-C, total cholesterol; TG, triglycerides; UCI, upper 95%
35 confidence interval; waist C, waist circumference; WHR, waist to hip ratio.

36

37

38 **Abstract**

39 Background. Exercise is recognized to evoke multisystemic adaptations that, particularly in
40 obese subjects, reduce body weight, improve gluco-metabolic control, counteract
41 sarcopenia and lower the risk of cardiometabolic diseases. Understanding the molecular
42 and cellular mechanisms of exercise-induced benefits is of great interest due to the
43 therapeutic implications against obesity.

44 Objectives and methods. The aim of the present study was to evaluate time-related changes
45 in size distribution and cell origin of extracellular vesicles (EVs) in obese and normal-weight
46 subjects who underwent a moderate-intensity exercise on a treadmill (at 60% of their
47 VO_{2max}). Blood samples were drawn before, immediately at the end of the exercise and
48 during the post-exercise recovery period (3h and 24h). Circulating EVs were analyzed by a
49 nanoparticle tracking analysis and flow cytometry after labeling with the following cell-
50 specific markers: CD14 (monocyte/macrophage), CD61 (platelet), CD62E (activated
51 endothelium), CD105 (total endothelium), SCGA (skeletal muscle) and FABP (adipose
52 tissue).

53 Results. In all subjects, acute exercise reduced the release of total (i.e., 30-700 nm) EVs in
54 circulation, predominantly EVs in the microvesicle size range (i.e., 130-700 nm EVs). The
55 post-exercise release of microvesicles was higher in normal-weight than obese subjects;
56 after exercise, circulating levels of exosomes (i.e., 30-130 nm EVs) and microvesicles were,
57 respectively, lower and higher in females than males. In all experimental subgroups (males
58 vs. females and obese vs. normal-weight subjects), acute exercise reduced and increased,
59 respectively, CD61+ and SCGA+ EVs, being the effect on CD61+ EVs prolonged up to 24h
60 after the end of the test with subjects in resting conditions. Total EVs, exosomes and CD61+
61 EVs were associated with HOMA-IR.

62 Conclusions. Though preliminary, the results of the present study show that a single bout of
63 acute exercise modulates the release of EVs in circulation, which are tissue-, sex- and BMI
64 specific, suggesting that the exercise-related benefits might depend upon a complex
65 interaction of tissue, endocrine, and metabolic factors.

66

Introduction

Exercise is reported to induce multisystemic benefits in humans, including decreasing cardiovascular risk [1, 2], improvement of glucometabolic homeostasis [3, 4], promotion of weight loss [5], counteracting sarcopenia [6, 7] and stimulation of anabolic hormones [8-10] in obese and/or type 2 diabetic patients. In particular, to understand the "obesity paradox" that higher body mass index (BMI) is associated with lower morbility/mortality, mainly in the geriatric population, it is important to take muscle mass into account rather than adiposity. In fact, there is evidence that sarcopenia with obesity (i.e., sarcopenic obesity) may be associated with higher levels of metabolic disorders and an increased risk of mortality than obesity or sarcopenia alone [11]. Therefore, our rehabilitative efforts to promote exercise in obese subjects should focus on both preventing obesity and maintaining or increasing muscle mass [12].

Anyway, uncertain are the molecular mechanisms governing the interaction between exercising skeletal muscle and the other organs, such as adipose tissue, endothelium and immune system, from which the exercise-induced cardiometabolic benefits derive.

There is robust evidence that exercise induces physiological and biochemical adaptations through the action of exerkines on target organs [13, 14], which can be distant from the site of production/release, mimicking the typical organization of the endocrine system [15].

Most biomolecules are extremely labile, easily inactivable by proteases and RNAases present in the plasma. In order to counteract this inhospitable environment, a very sophisticate system of extracellular trafficking and cell-to-cell targeting has been organized, i.e., the extracellular vesicles (EVs), including in particular exosomes and microvesicles. These are vacuolar structures, membrane-covered, containing proteins, nucleic acids (also of mitochondrial origin) and metabolites, which are implied in a wide range of physiological and pathological processes [16].

In particular, muscle-derived EVs have been shown to transfer exerkines of different chemical structure among cells and tissues [17, 18]. Furthermore, circulating levels of EVs change after exercise, having been produced and released by different tissues (not exclusively skeletal muscle) [19]. Type, intensity, and duration of exercise can modify size distribution and cell origin of EVs [20, 21]. There is the intriguing hypothesis that exercise-induced benefits, including improvement of glucometabolic homeostasis, decrease in cardiovascular risk and conversion/distribution of adipose tissue, are mediated by exosomes

99 and/or microvesicles, which may act in an autocrine, paracrine, and endocrine manner,
100 transferring exerkines from cell to cell [15].

101 One might hypothesize that EVs, native or pharmacologically modified, enriched with
102 specific exerkines endowed with antidiabetogenic, antisarcopenic, and antiobesogenic
103 properties, will represent, in future, a new therapeutic option for obesity-related
104 comorbidities [14, 19]. In this context, the recent demonstration of a vesiculogenic hyper-
105 responsiveness in obese subjects to environmental exposure (particularly, particulate
106 matter, PM) with a (presumptive) increase in thrombotic and, in general, cardiovascular risk
107 [22], suggests the existence of a "bad" vesiculogenic profiling, potentially changeable with
108 adequate therapeutic intervention, including constant execution of moderate exercise [23].

109 Therefore, based on the previous considerations, evaluation of vesiculogenesis in obese
110 (vs. normal-weight) female/male subjects undergoing an acute bout of exercise might be of
111 great interest. So, taking into account the tissues on which exercise exerts beneficial effects
112 (such as endothelium, muscle, adipose tissue and the immune system) cytofluorimetric
113 characterization of cell-specific EVs could be useful. In particular, this approach could help
114 to set the protocols of metabolic rehabilitation adopted in the multidisciplinary integrated
115 programs of body weight reduction. Furthermore, these knowledges could help in
116 understanding the pathophysiological mechanisms underlying the "altered" vesiculogenic
117 responsiveness in obese subjects to cardiometabolic factors [24] and environmental stimuli
118 [22] .

119

120 **Material and methods**

121

122 Subjects and protocol

123 Obese subjects (BMI > 40 kg/m^2), hospitalized at the Istituto Auxologico Italiano, Piancavallo
124 (VB), Italy, to take part of a multidisciplinary integrated program of body weight reduction,
125 were recruited for the current study. Normal-weight healthy subjects, age-matched, selected
126 among friends and relatives of the medical and nursing staff, were recruited as control group.
127 Both obese and normal-weight subjects were moderately active (60 min of physical activity,
128 two-times/week). All females were eumenorrheic; the study was carried out in the follicular
129 phase of their menstrual cycle.

130 After having verified exclusion criteria, particularly the existence of any disease, including
131 blood hypertension and type 2 diabetes mellitus, apart from morbid obesity, or assumption
132 of any drug, clinical, biochemical, and anthropometric data were collected from each
133 participant, including evaluation of body composition by bioimpedance analysis (Human-IM
134 Scan, DS-Medigroup, Milan, Italy). The test (described below) was administered after at
135 least 5 days of accommodation/admission at Piancavallo, where there are very low levels of
136 air pollution, in order to avoid the confounding factor of the environmental exposures on
137 vesiculogenesis such as PM [22].

138 Each subject underwent, in two different days (08.30-09.30AM), the following exercise
139 protocols::

140 PILOT TEST. At the beginning of the study, each participant performed an incremental
141 exercise on a treadmill (Technogym, Gambettola, Italy) until voluntary exhaustion; in
142 particular, after 3 min of resting, the subject performed 2 min of walking at 4 km/h and 0%
143 of slope, followed by speed increments of 0.5 km/h for each min up to 6 km/h; subsequent
144 slope increments were of 1% for each min up to 15%. Exhaustion was defined when one of
145 the following criteria was reached: 1) maximal levels (higher than 10) of self-perceived
146 exertion, using the Borg's modified CR10 scale [25] or 2) heart rate (HR) values higher than
147 90% of the age-predicted maximum. Data collected during the incremental test, including
148 VO_{2max} (Tab. 1) by indirect calorimetry (CPX Express, Medical Graphics Corp, MN) were
149 utilized to set the intensity of the submaximal test.

150 SUBMAXIMAL TEST. An exercise at a moderate constant workload, corresponding to 60%
151 of the aerobic threshold (VO_{2max}), established during the pilot test (see above), was
152 maintained for 30 min or until voluntary exhaustion. Obese subjects reached a HR peak of
153 170.5±9.9 beats per minute (bpm), while normal-weight subjects a HR peak of 166.7±13.9
154 bpm, without any significant difference.

155 Four blood samples, referred to the submaximal test, were drawn from an antecubital vein
156 of the arm by venipuncture: 1h before exercise (basal), immediately at the end of the
157 exercise (T0), 3h after (T3) and in the next day at the same time when the test was performed
158 (T24). While EV characterization (for size and cell origin) was performed in all time points,
159 biochemical parameters were measured only in the basal sample (see Supplementary
160 Material for details). Participants were permitted to consume comparable daily meals (i.e.,
161 lunch and dinner of the experimental day), which were strictly supervisioned by a nutritionist
162 for food composition (about 21% proteins, 53% carbohydrates and 26% lipids) and energy

163 intake (adherence); the submaximal test (at the experimental day) and the last blood
164 sampling (in the next day) were performed after 12h fasting.

165

166 <u>Characterization of size distribution and cell-origin of EVs</u>

167 Blood was collected into tubes containing EDTA and centrifuged at 1200 × g for 15 min at
168 room temperature to obtain platelet-free plasma, which was transferred in temperature-
169 controlled conditions (+4 C) to the EPIGET Lab (University of Milan) from Istituto Auxologico
170 Italiano within 3-27h after blood sampling. A detailed description of the method of EVs
171 isolation and purification from plasma is reported in Supplemetary Material.

172 Count and size of EVs were assessed by NTA (nanoparticle tracking analysis), a technique
173 that measures the Brownian motion of vesicles suspended in fluid which are displayed in
174 real time through a high sensitivity CCD camera.ith. Using a NanoSight LM10-HS system
175 (Amesbury, UK), EVs were detected by laser light scattering. Five 30-s recordings were
176 made for each sample. Collected data were analyzed with NTA software, which provides
177 high-resolution particle-size distribution profiles and concentration measurements of the
178 EVs (count/ml in plasma).

179 Cell origin of EVs was characterized by flow cytometry (MACSQuant, Miltenyi Biotec)
180 according to a standardized protocol [22]. Shortly, fluoresbrite® Carboxylate Size Range Kit
181 I (0.2, 0.5, 0.75, and 1 μm) was used to set the calibration gate on the analyzer. To analyze
182 EV integrity, 60 μl aliquots were stained with 0.02 μM 5(6)-carboxyfluorescein diacetate N-
183 succinimidyl ester (CFSE) at 37 °C for 20 min in the dark. Each aliquot of CFSE-stained
184 sample was incubated with a specific antibody: CD14-APC (clone TÜK4) for
185 monocyte/macrophage-derived EVs, CD105-APC (clone 43A4E1) for total endothelium-
186 derived EVs, CD62E (clone REA280) for activated endothelium-derived EVs, CD61-APC
187 (clone Y2/51) for platelet-derived EVs (Miltenyi Biotec, Bergisch Gladbach, Germany), A-
188 FABP (clone B-4) for adipocyte-derived EVs, and α-sarcoglycan SCGA (clone F-7) for
189 skeletal muscle-derived EVs (Santa Cruz Biotechnology, Dallas, Texas, USA). Before use,
190 each antibody was centrifuged at 17000 × g for 30 min at 4 °C to eliminate aggregates. The
191 stained PBS control sample was used to detect the autofluorescence of the antibody.
192 Quantitative multiparameter analysis of flow cytometry data was carried out by using FlowJo
193 Software (Tree Star, Inc.).

194

195 Statistical analysis

196 Standard descriptive statistics were performed on all variables. Continuous data are
197 expressed as mean ± standard deviation (SD), while categorical data are presented as
198 frequencies and percentages. Box plots were used to represent counts of EVs and cell-
199 specific EVs.

200 The Wilcoxon rank-sum test was used to compare, at baseline, demographics, clinical and
201 biochemical characteristics between obese and normal-weight subjects.

202 Since the aim of the study was to evaluate time-related changes in size distribution
203 (analyzed as the following size ranges: 30-130 nm or exosomes, 130-700 nm or
204 microvesicles and 30-700 nm or total EVs) and cell origin of EVs (i.e., CD14+, CD61+,
205 CD62E+, CD105+, SCG+, and FABP+) in obese and normal-weight subjects, having
206 outcome variables expressed as concentration (count/ml), a Poisson regression model for
207 count data was applied to determine the associations between exercise (basal, T0, T3 and
208 T24) and EVs or cell origins. The absence of over-dispersion was tested by the likelihood
209 ratio test. All potential confounders were included in the multivariate model after having
210 verified the presence of an association in a univariate model. Best model selection was
211 based on the minimization of the Akaike information criterion and maximization of the
212 explained variance of the model. The final models were adjusted for sex (F/M), BMI
213 (obese/normal-weight) and HOMA-IR. Other variables, including smoking, duration of
214 exercise, systolic and diastolic blood pressures, heart rate, waist to hip ratio (WHR), hCRP,
215 HbA_{1C}, LDL-C, HDL-C, T-C and TG, were additionally considered and then excluded in the
216 final model as their contribution to explain variance was not relevant.

217 We further analyzed the effect of exercise in terms of distribution of vesicle mean
218 concentrations for each EV size. For each EV size: 1) we estimated EV mean concentration
219 and 95% confidence interval (CI) at each time with unadjusted Poisson linear regression
220 models; 2) we compared the EV mean differences at each post-exercise time with respect
221 to basal; 3) we calculated q-FDR values using the multiple comparison methods based on
222 Benjamini-Hochberg False Discovery Rate (FDR), which takes into account the high number
223 of comparisons. As we observed different sex- and BMI patterns, the study population was
224 stratified in four groups according to BMI (obese/normal-weight) and sex (M/F). Results were
225 reported as a series graph for EV mean concentrations of each size and vertical bar charts
226 to represent the three p- and q- (i.e., FDR p-) values obtained comparing T0, T3 or T24 vs.
227 basal. For all the graphs X axis was the size of EVs.

228 A p-value <0.05 was considered statistically significant.

229 All analyses were run using SAS Software (version 9.4, Cary, NC: SAS Institute. Inc.)

230

231 **Results**

232 Study population included fifteen obese (F/M = 8/7) and eight normal-weight healthy
233 subjects (F/M = 4/4). Main characteristics of the subjects, subdivided into normal-
234 weight/obese and female/male groups, are reported in Tab. 1.

235 Boxplots describing time-dependent release of exosomes (i.e., 30-130 nm EVs),
236 microvesicles (i.e., 130-700 nm EVs) and total EVs (i.e., 30-700 nm EVs) are reported in
237 Fig. 1. Comparisons of the adjusted means of these EVs among the time points of the
238 protocol (i.e., basal, T0, T3, and T24) are summarized in Tab. 2.

239 In particular, after adjusting the models for sex, BMI, HOMA-IR and time, the total EVs after
240 exercise significantly decreased (p=0.045), being the adjusted means of these EVs
241 significantly reduced immediately at the end of the exercise and after 3h and 24h (p= 0.013,
242 p= 0.001 and p= 0.013 vs. basal, respectively) (Tab. 2). A similar significant decrease in
243 microvesicles occurred after exercise (p=0.008), being the adjusted means of these EVs
244 significaly reduced after 3h and 24h (p<0.001 vs. basal for both time pints) (Tab. 2). The
245 effect of exercise on exosomes was not significant (p= 0.265); anyway, exosomes
246 immediately after the end of the exercise were significantly lower than those at the basal
247 (p=0.042) (Tb. 3). There was a significantly higher post-exercise release of microvesicles in
248 normal-weight than obese subjects (p= 0.036). Furthermore, a significant association of
249 microvesicles with sex was found, being the post-exercise release of these EVs higher in
250 females than males (p= 0.033). When considering the association of exosomes with sex,
251 there was a significantly lower release of these EVs in females than males (p< 0.042). No
252 significant association of exosomes with BMI was found.

253 Fig. 2 shows, in a whole view, without any pooling for size ranges, the effect of exercise in
254 terms of distribution of vesicle mean concentrations for each EV (single) size. As different
255 sex- and BMI-related patterns were present, the study population was stratified in four
256 groups: i) normal-weight females (NW-F, panel A); ii) normal-weight males (NW-M, panel
257 B); iii) obese females (OB-F, panel C); iv) obese males (OB-M, panel D). In particular, as
258 shown by the upper part of each panel of Fig. 2, corresponding to one of these patients'

259 groups, exercise produced evident changes in the mean concentrations calculated for each
260 EV size, ranging from 30 to 700 nm. The comparisons among the EV sizes are shown in the
261 lower part of each panel of Fig. 2, where the three p- and q- (i.e., FDR p-) values obtained
262 comparing T0, T3 or T24 vs basal are reported.

263 Comparisons of the adjusted means of cell-specific EVs (i.e., CD14+, CD61+, CD62E+,
264 CD105+, SCG+ and FABP+ EVs) among the four-time points of the protocol (basal, T0, T3,
265 and T24) are summarized in Tab. 3. Fig. 3 reports boxplots describing time-dependent
266 releases of CD61+, and SCG+ EVs.

267 In particular, after exercise, CD61+ EVs (i.e., platelet derived EVs) significantly decreased
268 ($p = 0.025$), being the adjusted mean of these EVs significantly reduced after 24h ($p< 0.001$
269 vs. basal), with the counts at the other time points not significantly different vs. basal (Tab.
270 3). No significant association of CD61+ EVs with BMI was found; on the contrary, there was
271 a significant higher post-exercise release of these EVs in females than males ($p= 0.037$).

272 The effect of exercise on SCGA+ EVs (i.e., skeletal muscle-derived EVs) was not significant
273 ($p= 0.121$); nevertheless, there was a significant post-exercise increase in the adjusted
274 mean of these EVs immediately after the end of the exercise ($p= 0.016$ vs. basal), with the
275 counts at the other time points not significantly different vs. basal (Tab. 3). No significant
276 associations of SCGA+ EVs with sex or BMI were found.

277 There were no significant associations of CD14+, CD62E+, CD105+ and FABP+ EVs (i.e.,
278 monocyte/macrophage-, activated endothelium-, total endothelium-, adipose tissue-derived
279 EVs, respectively) with exercise, sex, and BMI.

280 Total EVs, exosomes and CD61+ EVs were significantly associated with HOMA-IR (β=
281 0.123±0.044, p=0.005; β=0.183±0.054, p=0.001; β=-0.123±0.044, p=0.005; β=0.238±0.097,
282 p=0.014, respectively).

283

284

285 **Discussion**

286 The results of the present study, carried out in obese and normal-weight females and males
287 undergoing a single bout of acute exercise, show that: (1) acute exercise is capable of
288 decreasing the release of (total, i.e., 30-700 nm) EVs in circulation, acting predominantly on
289 the microvesicle-enriched fraction (i.e., 130-700 nm EVs); (2) the post-exercise release of

290 microvesicles (i.e., 130-700 nm EVs) is higher in normal-weight than obese subjects; (3) the
291 post-exercise releases of exosomes (i.e., 30-130 EVs) and microvesicles (i.e., 130-700 nm
292 EVs) are, respectively, lower and higher in females than males; (4) in all experimental
293 subgroups (males vs. females and obese vs. normal-weight subjects), acute exercise
294 reduces and increases, respectively, CD61+ EVs (i.e., those deriving from platelets) and
295 SCGA+ EVs (i.e., those deriving from skeletal muscle), being the effect on CD61+ EVs
296 prolonged up to 24h after the end of the test with subjects in resting conditions; (5) total (i.e.,
297 30-700 nm) EVs, exosomes (i.e., 30-130 nm EVs) and CD61+ (i.e., platelet-derived EVs)
298 are associated with HOMA-IR.

299 The major limitation of our study (present in most of the studies similar to ours) was the
300 tissue characterization of EVs by using only a restricted panel of cell-specific antibodies.
301 Furthermore, one tissue can release many EVs, phenotypically and functionally different,
302 presumably characterizable with a variety of antibodies [26]. For example, platelet-derived
303 EVs can be CD61+, but not all platelet-derived EVs are likely to be CD61+ [28].

304 Based on the previous considerations, our finding that acute exercise provokes marked
305 changes in sizes and counts of plasma EVs irrespectively from the characterization of cell
306 origin suggests that an enormous variety of EVs, deriving from different tissues or from the
307 same tissue with a different phenotype, is affected by exercise [21]. Further studies are
308 needed to characterize these EVs mainly in terms of cell origin, an obligatory step before
309 identifying the cargo of biomolecules inside each type of EVs and understanding their
310 function [14].

311 In the present study, while there was no post-exercise effect on exosomes (i.e., 30-130 nm
312 EVs) apart from the significant *post-hoc* comparison immediately after the end of the
313 exercise (vs. basal). the release of plasma total (i.e., 30-700 nm) EVs and microvesicles
314 (i.e., 130-700 nm EVs) decreased at 3h and 24h after exercise. Total EVs also decreased
315 immediately after the end of the exercise. Analyzing the counts of EVs for size and cell
316 origin, these results cannot be numerically explained by the decrease of platelet-derived
317 EVs (i.e., those CD61+), which, though prevalently microvesicles, represent only a minimal
318 fraction. We can also rule out the contributions of the other EVs, characterized by flow
319 cytometry with the antibodies used in the present study, being their plasma counts
320 unchanged after exercise (particularly, endothelium-, adipocyte and monocyte/macrophage-
321 derived EVs). Because some cell-specific EVs could increase after exercise (see below for
322 SGCA+ EVs), this search becomes more difficult.

323 Sex-related differences in post-exercise release of EVs were observed in the present study:
324 in particular, after exercise, exosomes (i.e., 30-130 nm EVs) were lower in females than
325 males, while microvesicles (i.e., 130-700 nm) were higher in females than males. Although
326 post-exercise release of EVs in females and males has been investigated in only a few
327 studies [28, 29], sex hormones are likely to be responsible of this sex-related difference as
328 suggest by the effects of menstrual cycle on (resting) vesiculogenesis [30].

329 Unfortunately, we were unable to characterize the cell origin of the EVs differently present
330 in females vs. males, being negligible the higher post-exercise contribution of CD61+ EVs
331 (i.e., platelet-derived EVs) in females than males of our study. In this context, some other
332 studies have demonstrated a sex dismorphism in the post-exercise release of
333 (CD31+/CD42b-) platelet- and (CD62E+) endothelium-derived EVs, an effect which also
334 dependson exercise intensity[28, 29].

335 Although conflicting results have been reported, obese subjects generally exhibit increased
336 plasma levels of EVs in resting conditions (~ 10 fold when compared to a normal-weight
337 group), being the size profiling of the EVs formed by exosomes (20%) and microvesicles
338 (80%) [31]. In contrast, in the present study, a BMI-related difference in post-exercise
339 vesiculogenesis was found, being the release of microvesicles (130-700 nm EVs) higher in
340 normal-weight than obese subjects undergoing the same exercise test, As there were no
341 BMI-related differences in post-exercise decrease in total (i.e., 30-700 nm) EVs and CD61+
342 EVs and increase in SCGA+ EVs, based on the results of the present study, EVs deriving
343 from other tissues should be detected in future studies to determine the predominant tissue
344 source of the different vesiculogenesis in obese vs. normal-weight subjects at rest and after
345 an acute stimulus such as exercise.

346 Though acute exercise did not change the release of FABP+ EVs (i.e., adipocyte-derived
347 EVs), we hypothesize that the post-exercise vesiculogenic responsiveness of obese
348 subjects, when compared to the normal-weight counterpart, is due to “metabolic” factors to
349 be identified. In this context, it is noteworthy that HOMA-IR was associated with post-
350 exercise releases of total (i.e., 30-700 nm) EVs, exosomes and CD61+ EVs. The link
351 between glucometabolic homeostasis and vesiculogenesis may be of great interest to
352 understand the molecular and cellular mechanisms underlying the well-known
353 antidiabetogenic effect of exercise in obese subjects [3, 4]. Given that obese patients are
354 frequently insulin-resistant (a condition not occurring in the present study), an (intriguing)
355 hypothesis is that it may not be obesity *per se*, but rather insulin-resistance that produces a

356 different post-exercise response in EVs between obese vs. normal-weight groups. So, one
357 might argue that a metabolically controlled obese subject obtains better cardiometabolic
358 benefits from exercise. Indeed, the effectiveniess of some multidisciplinary integrated
359 programs of body weight reduction, in which exercise is combined with other interventions
360 such as diet and pharmacotherapy, might derive from the relationship between
361 vesiculogenesis and insulin-resistance/sensitivity. Further studies are mandatory to confirm
362 this hypothesis.

363 SCGA (i.e., sarcoglycan α) is a component of the dystrophin-glycoprotein complex, which,
364 being implicated in type 2D autosomal recessive limb-girdle muscular dystrophy, has been
365 supposed to play a fundamental role in the stability of muscle fiber membranes and in the
366 connection of cytoskeleton to the extracellular matrix [32]. The gene *scga* is strictly
367 expressed in striated skeletal muscle [33]. Post-exercise release of SCGA+ EVs has been
368 already reported in humans together with the identification of a specific mRNA cargo [34].

369 To our best knowledge, the present study is the first to show that there is a post-exercise of
370 SCGA+ EVs in obese subjects, too, a response that was similar to that in normal-weight
371 subjects, with no differences in females and males for both BMI subgroups.

372 We are not aware whether there is an alteration in muscle vesiculogenesis in sarcopenic
373 obesity, as our obese subjects were young with no clinical signs of sarcopenia. So far, this
374 topic has been never investigated. Anyway, if SCGA+ EVs are supposed to transfer a cargo
375 of biomolecules endowed with "myoregulatory" function such as myomiRs [34], any effort
376 should be made to maximally stimulate the release of SCGA+ EVs, which, in autocrine and
377 paracrine manner, could contrast (or prevent) sarcopenia in obesity [15]. Therefore, the
378 choice of an exercise in terms of type, intensity, and duration is fundamental when setting
379 rehabilitative programs for sarcopenic obese subjects [19]. Further studies in exercising
380 sarcopenic and non-sarcopenic obese subjects are mandatory to confirm our hypothesis
381 regarding the physiological and also therapeutic role of SCGA+ EVs.

382 Differently from the results of other studies, in which no difference or an increase in post-
383 exercise platelet-derived EVs (particularly, CD41a+ or CD42b+ EVs) was found [19, 35, 36],
384 a decrease in CD61+ EVs occurred in our study population after acute exercise.
385 Interestingly, this effect was more evident at 24h after the end of the test.

386 The physiological or pathophysiological role of CD61+ EVs, simply platelet-derived EVs, is
387 only partially known [37]. There is some evidence that these EVs, including their cargo of

388 biomolecules, are implicated in thrombotic, atherogenic and inflammatory processes [38].
389 So, one might argue that the exercise-induced beneficial effects on the cardiovascular
390 system are mediated, at least in part, by an inhibition of platelet vesiculogenesis. As the
391 protective effect of exercise on the cardiovascular system has been also demonstrated in
392 healthy normal-weight subjects [39], our finding that both normal-weight and obese subjects
393 exhibited a similar post-exercise decrease in the release of CD61+ EVs supports the notion
394 that a common molecular and cellular mechanism underlies the beneficial effects of exercise
395 in all individuals with or without cardiometabolic diseases. Nevertheless, when exercise is
396 strenuously executed at high intensity and for long times, the cardiovascular risk seems to
397 increase, mainly due to a hypercoagulative state [40], which has been associated to a post-
398 exercise increase of plasma platelet-derived EVs in normal-weight subjects [41].

399 In the present study, use of antibodies for other cell-specific markers, i.e., CD14, CD62b,
400 CD105, and FABP, evidenced no changes in the post-exercise releases of
401 monocyte/macrophage-, activated/resting endothelium- and adipocyte-derived EVs,
402 respectively. As conflicting results have been reported in literature [19], we should identify
403 the reasons of this discrepancy. Methodological differences, including the specificity of the
404 antibodies, characteristics of the exercise test administered to the recruited subjects (type,
405 intensity and duration), times of blood sampling, and statistical pitfalls such as sample size
406 might be the most relevant reasons to be taken into account. As post-exercise
407 vesiculogenesis is crucial in understand molecular and cellular mechanisms underlying
408 exercise-induced beneficial effects in special populations, particularly obese subjects [15],
409 further studies, including those carried out in *in-vitro* models consisting of specific cell lines,
410 are needed to solve this issue.

411 In conclusion, the results of the present study show that a single bout of acute exercise
412 induces changes in the release of EVs in circulation, which are tissue-, sex- and BMI
413 specific, suggesting that the exercise-related benefits might depend upon a complex
414 interaction of tissue, endocrine, and metabolic factors [14, 19, 42].

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Authors' contribution statement

A. E. Rigamonti, together with V. Bollati and A. Sartorio designed the study. L. Abbruzzese, A. De Col, S. Tamini and S. Cicolini enrolled the subjects and performed the tests. R. De Micheli and G. Tringali performed the evaluation of body composition of all patients. A. De Col performed the biochemical determinations, while L. Pergoli isolated and characterized plasma EVs. S. Tamini, A. De Col and S. Cicolini elaborated the database. S. Iodice and A. E. Rigamonti analyzed the data. A. E. Rigamonti, together with A. Sartorio, wrote the manuscript. V. Bollati and S. G. Cella contributed to data interpretation and discussion writing. All authors contributed to the manuscript revision.

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The patients and their parents (for the subjects younger than 18 yrs) and the healthy controls were fully informed of the procedures and possible risks associated with the experiments before giving their written consent to participate to the study. The protocol was approved by the local ethics committee (reference code: 01C825-2018; acronym: VESCIOBES). All procedures were in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

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565

566

567 **Legend to figures**

568

569 Fig. 1. Boxplot of post-exercise releases of plasma exosomes (30-130 nm EVs),
570 microvesicles (130-700 nm EVs) and total EVs (30-700 nm EVs) (for all data). The vertical
571 line inside the box is the median (50th percentile); the two vertical lines that constitute the
572 top and bottom of the box are the 25th and 75th percentiles, respectively; the whiskers are
573 calculated as ±1.5× IQR; finally, the outliers are drawn as a small symbol. Mean EVs
574 concentrations on Y axis are expressed as 10^8 count/ml. Asterisks indicate significant
575 comparisons between adjusted mean EVs at each post-exercise time point vs. basal value.
576 * $p<0.05$; ** $p<0.01$. Note that p-values were calculated from multivariable linear models
577 adjusted for sex, BMI (obese/normal-weight), HOMA-IR and time.

578

579 Fig. 2. The top of each panel reports size profiling of plasma EVs at the nanoparticle tracking
580 analysis (NTA) for each time of blood sampling (B, T0, T3 and T24) after a single bout of
581 acute exercise. The study population was stratified in four groups: i) normal-weight females
582 (NW-F, panel A); ii) normal-weight males (NW-M, panel B); iii) obese females (OB-F, panel
583 C); iv) obese males (OB-M, panel D). The bottom of each panel reports the p- (dark dots)
584 and q- (shaded dots) values of comparisons of EVs for the entire 30-700 nm size range at
585 one post-exercise time point (i.e., T0, T3 or T24) vs. basal (B).

586

587 Fig. 3. Boxplot of post-exercise releases of plasma CD61+ EVs (i.e., platelet-derived EVs)
588 (left panel) and SCGA+ EVs (i.e., muscle-derived EVs) (right panel). The vertical line inside
589 the box is the median (50th percentile); the two vertical lines that constitute the top and
590 bottom of the box are the 25th and 75th percentiles, respectively; the whiskers are calculated
591 as ±1.5× IQR; finally, the outliers are drawn as a small symbol. Asterisks indicate significant
592 comparisons between adjusted mean EVs at each post-exercise time point vs. basal value.
593 * $p<0.05$; ** $p<0.01$. Note that p-values were calculated from multivariable linear models
594 adjusted for sex, BMI (obese/normal-weight), HOMA-IR and time.

595

596

Tab. 1. Demographic, clinical and biochemical characteristics of the study population, subdivided in normal-weight/obese and female/male groups.

Parameter	*Obese*		*Normal-Weight*	*p-value*			
	All Data	*Female*	*Male*	*All Data*	*Female*	*Male*	
N.	15	8	7	8	4	4	-
Age (yr)	21.2±8.8	21.0±10.1	21.4±7.8	26.2±7.2	20.5±3.5	31.8±4.7	0.057
Weight (kg)	155.0±21.2	93.0±11.2	123.4±18.1	65.2±11.2	57.1±6.5	73.4±8.4	0.000
Height (mt)	1.7±0.1	1.6±0.1	1.8±0.1	1.7±0.1	1.7±0.1	1.7±0.1	0.771
BMI (kg/m^2)	36.9±4.9	34.2±3.9	40.1±4.0	22.3±3.1	20.4±1.5	24.2±3.3	0.000
Waist C (cm)	107.8±16.8	96.7±7.3	120.6±15.6	80.8±7.4	75.8±5.3	85.8±5.8	0.000
Hip C (cm)	122.1±9.1	121.1±8.1	123.3±10.8	94.3±6.2	94.8±6.6	93.8±6.7	0.000
WHR	0.9±0.1	0.8±0.1	1.0±0.1	0.9±0.1	0.8±0.0	0.9±0.0	0.675
HR (beat/min)	93.1±14.7	99.0±11.8	86.3±15.5	80.5±4.3	82.5±1.9	78.5±5.4	0.042
SBP (mmHg)	123.3±9.8	120.0±7.6	127.1±11.1	110.0±4.6	108.0±6.3	111.3±2.5	0.002
DBP (mmHg)	80.7±5.9	80.0±5.3	81.4±6.9	71.9±5.9	70.0±7.1	73.8±4.8	0.005
FFM (kg)	60.8±13.0	50.7±5.2	72.2±8.8	52.4±10.5	43.2±4.2	61.6±3.4	0.186
FFM (%)	56.7±5.5	54.9±6.0	58.8±4.3	80.2±6.8	76.1±5.8	84.4±5.3	0.000
FM (kg)	46.4±11.0	42.3±9.7	51.2±11.0	12.8±4.5	13.8±4.1	11.8±5.1	0.000
FM (%)	43.3±5.5	45.1±6.0	41.2±4.3	19.8±6.8	23.9±5.8	15.6±5.3	0.000
Glucose (mg/dl)	84.3±4.8	83.5±4.9	85.1±4.9	89.4±6.5	84.3±2.2	94.5±5.0	0.051
Insulin (mIU/l)	16.0±5.6	13.9±4.3	18.4±6.2	10.0±5.0	12.5±6.2	7.6±1.9	0.008
HOMA-IR	3.3±1.2	2.9±1.0	3.9±1.3	2.2±1.0	2.6±1.3	1.8±0.5	0.009
HbA_{1C} (%)	5.1±0.4	5.0±0.4	5.1±0.5	5.2±0.3	5.1±0.3	5.3±0.2	0.603
T-C (mg/dl)	150.6±32.4	147.0±36.5	154.7±29.4	178.9±30.6	194.0±30.1	163.8±25.8	0.053
HDL-C (mg/dl)	40.8±8.1	43.9±9.0	37.3±5.6	54.3±11.3	53.8±13.6	54.8±10.7	0.009
LDL-C (mg/dl)	98.5±27.1	95.8±29.9	101.7±25.4	116.4±29.9	130.3±21.4	102.5±33.5	0.185
TG (mg/dl)	105.3±44.2	92.3±33.0	120.1±53.0	84.4±43.0	105.3±55.7	63.5±7.1	0.076
CRP (mg/dl)	0.2±0.2	0.3±0.3	0.1±0.1	0.1±0.1	0.2±0.1	0.1±0.1	0.723
Smokers (n.)	5	2	3	3	2	1	-
VO_{2max} (l/min)*	2.4±0.7	1.9±0.3	2.9±0.5	2.0±0.7	1.6±0.3	2.3±0.8	0.087
VO_{2max} [ml/(min×kg)]*	23.3±4.9	22.9±5.5	23.8±4.5	29.7±5.1	28.9±3.2	30.4±7.0	0.006
ED (min)	1.1±7.3	17.9±7.2	24.9±5.8	28.1±3.7	28.8±2.5	27.5±5.0	0.024

Data are reported as mean±SD; Wilcoxon rank-sum test was used to compare obese and normal-weight subjects.
Abbreviations: BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; FFM, fat-free mass; FM, fat mass; HbA_{1C}, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; Hip C, hip circumference; HOMA-IR, homeostatic model assessment of insulin resistance; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; T-C, total cholesterol; TG, triglycerides; Waist C, waist circumference; WHR, waist to hip ratio. *: measured during the pilot test.
Please, note that waist circumference was measured in standing position halfway between the inferior margin of the ribs and the superior border of the crista, while hip circumference was measured as the greatest circumference around the nates.

Tab. 2. Basal and post-exercise release of plasma exosomes, microvesicles and total EVs.

Outcome	*EVs size*	*Time*	*Adj. means* ($\times 10^8$/ml)	*LCI*	*UCI*	*p-value*	*Slope p-value*
Evs	30 to 700 nm						0.045
		Basal	46.3	40.5	52.9	Ref.	
		T0	40.4	35.5	46.0	0.013	
		T3	36.9	3.1	42.5	0.001	
		T24	36.6	29.3	45.9	0.013	
Microvesicles	130 to 700 nm						0.008
		Basal	25.9	21.6	30.5	Ref.	
		T0	23.1	19.6	27.3	0.171	
		T3	17.8	15.2	20.9	0.000	
		T24	16.3	14.5	18.4	0.000	
Exosomes	30 to 130 nm						0.265
		Basal	19.1	16.1	22.8	Ref.	
		T0	16.0	13.3	19.2	0.042	
		T3	17.6	14.7	21.2	0.382	
		T24	18.7	14.3	24.5	0.831	

Models were adjusted for sex, BMI, HOMA-IR and time; *p*-value refers to comparisons between the adjusted mean EVs at each post-exercise time point vs. basal value; slope *p*-value refers to relationships between time and EVs.
Abbreviations: LCI, lower 95% confidence interval; UCI, upper 95% confidence interval; Ref, reference category used for comparing pairs of times.

Tab. 3. Basal and post-exercise release of cell-specific *EVs.*

Outcome	*Cell-specific origin*	*Time*	*Adj. means*	*LCI*	*UCI*	*p-value*	*Slope p-value*
CD105+ Evs	Total endothelium						0.263
		Basal	6.3	5.3	7.4	Ref.	
		T0	7.5	6.2	9.0	0.102	
		T3	6.3	5.1	7.7	0.960	
		T24	6.1	4.7	7.8	0.786	
CD14+ Evs	Monocyte/macrophage						0.312
		Basal	8.8	6.7	11.5	Ref.	
		T0	9.6	7.4	12.4	0.488	
		T3	7.6	5.6	10.4	0.225	
		T24	8.4	6.5	10.9	0.671	
CD61+ EVs	Platelet						**0.025**
		Basal	23.8	19.0	29.9	Ref.	
		T0	28.5	20.1	40.3	0.303	
		T3	19.3	12.9	28.9	0.279	
		T24	12.6	10.1	15.7	**<0.001**	
CD62E+ Evs	Activated endothelium						0.546
		Basal	8.8	7.2	10.8	Ref.	
		T0	8.9	7.2	10.9	0.961	
		T3	8.0	6.4	9.9	0.295	
		T24	8.2	6.6	10.3	0.371	
FABP+ EVs	Adipose tissue						0.915
		Basal	7.7	6.4	9.4	Ref.	
		T0	8.2	6.1	10.8	0.511	
		T3	7.7	5.6	10.5	0.946	
		T24	7.6	5.9	9.7	0.763	
SCGA+ EVs	Striated skeletal muscle						0.121
		Basal	9.3	7.4	11.7	Ref.	
		T0	11.9	9.4	15.0	**0.016**	
		T3	9.6	7.6	12.3	0.602	
		T24	11.6	8.9	15.0	**0.056**	

Note that *p*-value refers to comparisons between the adjusted cell-specific mean EVs at each post-exercise time point vs. basal; slope p-value refers to relationships between time and each cell-specific EVs.
Abbreviations: LCI, lower 95% confidence interval; UCI, upper 95% confidence interval; Ref, reference category used for comparing pairs of times.

Figure 1

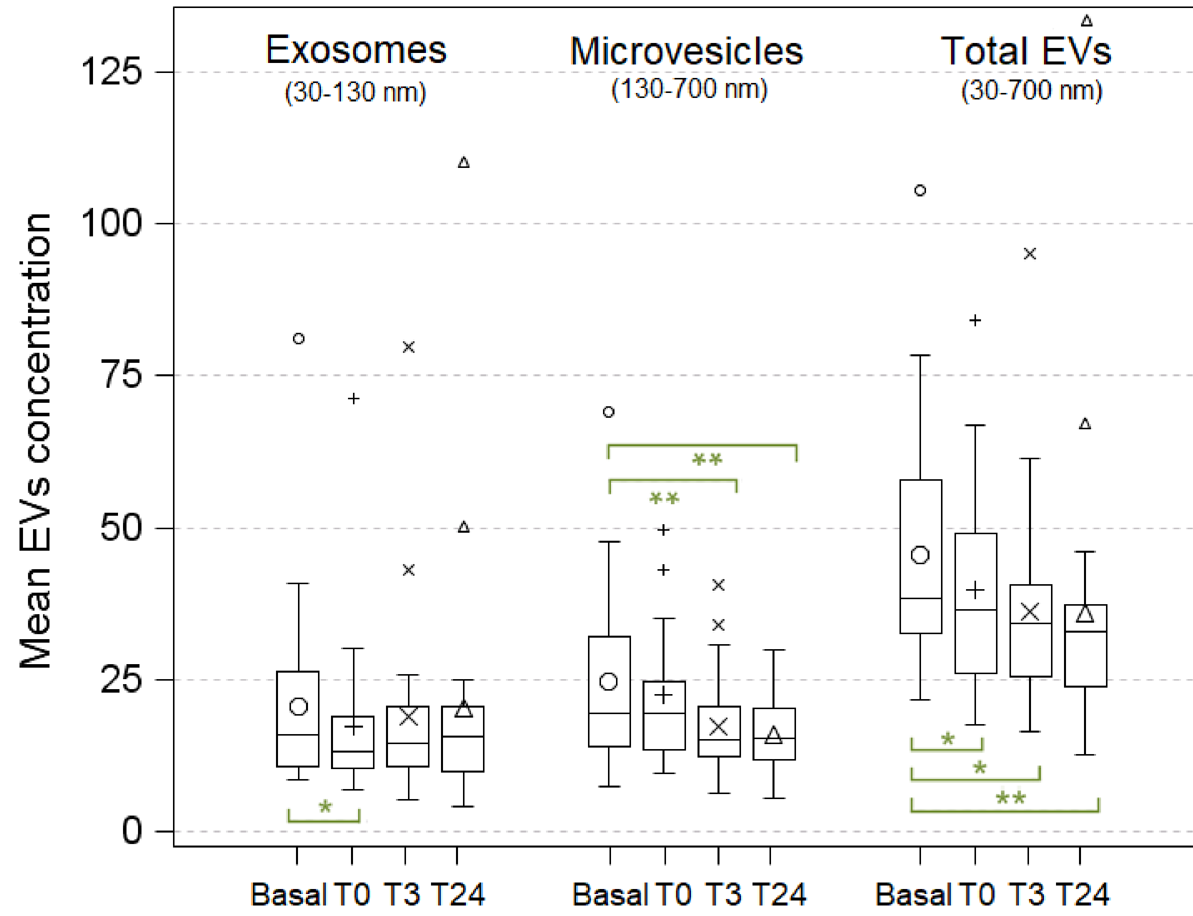


Figure 2

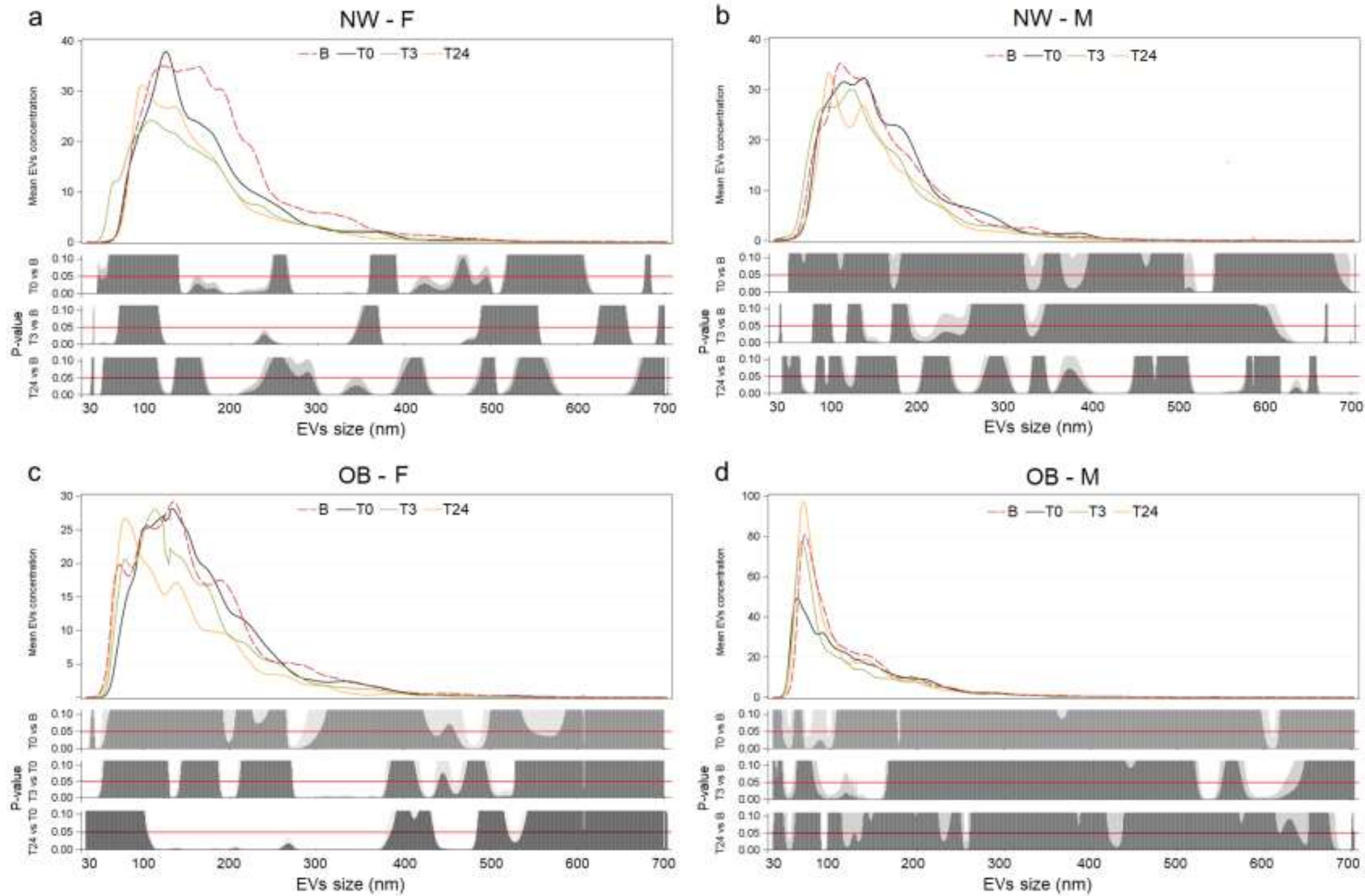
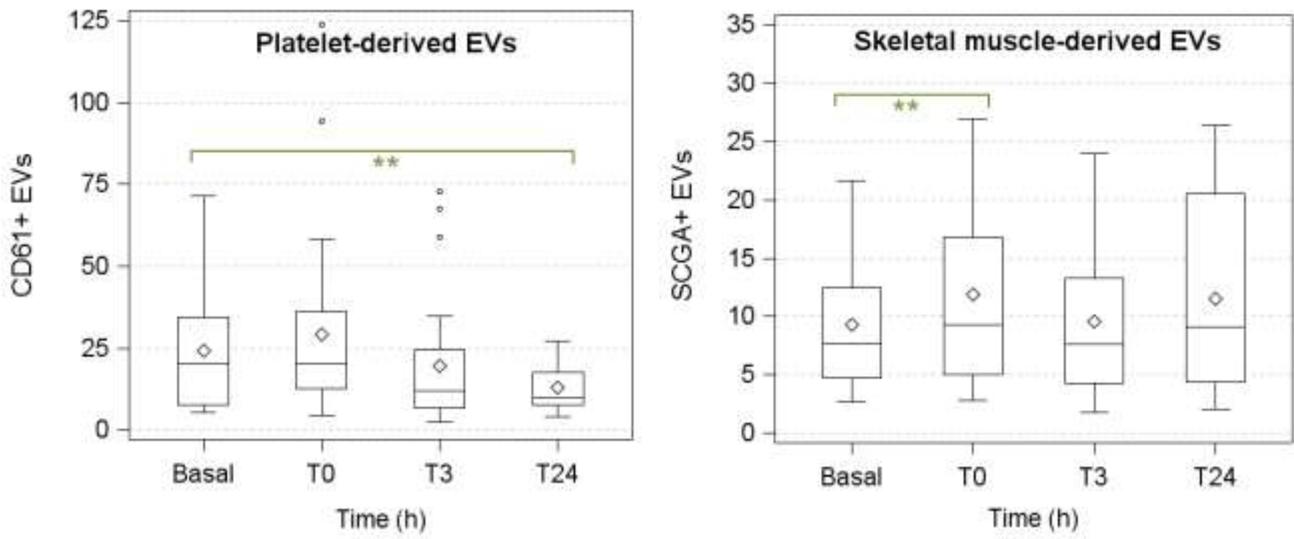


Figure 3



Supplementary material

Biochemical measurements

Serum insulin concentration was determined by a chemiluminescent immunometric assay, using a commercial kit (Elecsys Insulin, Roche Diagnostics, Monza, Italy). The sensitivity of the method was 0.2 μIU/ml [1 μU/ml = 7.18 pmol/l].

Serum glucose level was measured by the glucose oxidase enzymatic method (Roche Diagnostics, Monza, Italy). The sensitivity of the method was 2 mg/dl [1 mg/dl = 0.06 mmol/l].

Colorimetric enzymatic-assays (Roche Diagnostics, Monza, Italy) were used to determine serum total cholesterol (T-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) levels. The sensitivities of the assays were 3.86 mg/dl [1 mg/dl = 0.03 mmol/l], 3.87 mg/dl [1 mg/dl = 0.03 mmol/l], 3.09 mg/dl [1 mg/dl = 0.03 mmol/l] and 8.85 mg/dl [1 mg/dl = 0.01 mmol/l], respectively.

Glycated hemoglobin (HbA_{1C}) was measured by capillary electrophoresis, using a CAPILLARYS HbA_{1C} kit (Sebia, Bagno a Ripoli, Italy) on the CAPILLARYS 2 Flex-Piercing instrument.

Serum level of high-sensitive C-reactive protein (hsCRP) was determined by particle-enhanced turbidimetric immune-assay (Roche Diagnostics, Monza, Italy). The sensitivity of the method was 0.015 mg/dl [1 mg/dl = 95.24 nmol/l].

Estimation of insulin resistance

As an estimate of insulin resistance, fasting glucose and insulin levels were used to compute the homeostatic model assessment of insulin resistance (HOMA-IR), which was calculated in accordance with the following formula: [insulin (μIU/ml) × glucose (mg/dl)]/405.

Isolation and purification of EVs from plasma

Plasma was centrifuged at 1000, 2000, and 3000 × g for 15 min at 4 °C. The pellet was discarded to remove cell debris.

To prepare an EV pellet, 1.5 ml of fresh plasma was transferred to a 13.5 ml polypropylene ultracentrifuge tube (Beckman Coulter), which was filled with PBS, which was filtered through a 0.10 µm pore-size polyethersulfone filter (StericupRVP, Merck Millipore) to minimize the background contribution of interfering particles. Plasma was ultracentrifuged (Beckman Coulter Optima-MAX-XP) at 110000 × g for 75 min at 4 °C, to obtain an EV-rich pellet. The pellet was resuspended in 500 µl of triple-filtered PBS (0.10 µm pore-size).