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Antonela S Fittipaldi, Daniel Castrogiovanni, Daniela Lufrano, Camila Saenz, Pablo N de Francesco, Tyler Lalonde, Leonard G Luyt, Sonia Cantel, Jean-Alain Fehrentz, María F Andreoli, et al.

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## **Ghrelin Proteolysis Increases in Plasma of Men, but not Women, with Obesity**

**Running title:** ghrelin proteolysis in human obesity

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**Running title:** ghrelin proteolysis in human obesity

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34 **Abstract**

35

36 Aims: Since plasma ghrelin can undergo des-acylation and proteolysis, the aim of this study  
37 was to investigate the extent to which an enhancement of these reactions is associated to  
38 the decrease of ghrelin in plasma after food intake or in individuals with obesity.

39 Main methods: we performed an intervention cross-sectional study, in which levels of  
40 ghrelin, desacyl-ghrelin (DAG), glucose, insulin, ghrelin des-acylation and ghrelin  
41 proteolysis were assessed in plasma before and after a test meal in 40 people (n=21 males)  
42 with normal weight (NW, n=20) or overweight/obesity (OW/OB, n=20).

43 Key findings: Preprandial ghrelin and DAG levels were lower, whereas preprandial ghrelin  
44 proteolysis was ~4.6-fold higher in plasma of males with OW/OB. In males, ghrelin  
45 proteolysis positively correlated with glycemia. Ghrelin and DAG levels were also lower in  
46 females with OW/OB, but preprandial ghrelin proteolysis was not different between females  
47 with NW or OW/OB. Ghrelin and DAG levels decreased postprandially in males and females,  
48 independently of BMI, and ghrelin proteolysis increased postprandially ~2 folds only in  
49 individuals with NW. Ghrelin des-acylation remained unaffected by BMI or feeding status in  
50 both sexes.

51 Significance: Current study shows that ghrelin proteolysis increases in males with obesity  
52 as well as after meal in lean individuals. Therefore, ghrelin proteolysis may be an important  
53 checkpoint and, consequently, a putative pharmacological target to control circulating  
54 ghrelin levels in humans.

55

56 **Key words**: ghrelin proteolysis, ghrelin des-acylation, obesity.

## 57 Introduction

58

59 Ghrelin is a 28-residue peptide hormone predominantly secreted by cells of the  
60 stomach that displays several unique biochemical and biological features <sup>1</sup>. Chemically,  
61 ghrelin is the only peptide known to be acylated with an octanoic moiety, a posttranslational  
62 modification that takes place at Ser3 of ghrelin and is catalysed by the enzyme ghrelin-O-  
63 acyltransferase (GOAT) early in the biosynthetic processing within the ghrelin-producing  
64 cells <sup>2</sup>. The biological actions of ghrelin, which are mediated by the growth hormone  
65 secretagogue receptor (GHSR), entirely depend on the octanoylation of ghrelin and include  
66 the most potent orexigenic effect described to date <sup>3,4</sup>. Ghrelin also induces growth hormone  
67 and glucocorticoid secretion, recruits several mechanisms that increase glycemia, increases  
68 gastrointestinal motility and affects non-homeostatic aspects of food intake, among other  
69 effects <sup>1</sup>. Thus, ghrelin is a pleiotropic hormone that contains an exceptional  
70 posttranslational modification that is essential for its bioactivity.

71

72 Plasma ghrelin level is tightly regulated by the daily feeding status and by the long-  
73 term energy balance. In the short term, ghrelin level increases before meals and decreases  
74 to baseline levels within the first hour after meals <sup>5</sup>. Ghrelin level increases in prolonged  
75 energy deficit conditions, such as fasting, calorie restriction or anorexia nervosa <sup>6,7</sup>, when a  
76 rise in plasma ghrelin contributes to seek for food and maintain glycemia. In contrast, ghrelin  
77 level decreases under energy surplus conditions, such as obesity <sup>8,9</sup>, and such decrease  
78 presumably results as a compensatory adaptation aiming to reduce feeding and glycemia.  
79 As other hormones, plasma ghrelin level depends on the equilibrium among its secretion,  
80 degradation and clearance rates. A large body of studies characterized the mechanisms  
81 controlling the biosynthesis and release of ghrelin from ghrelin cells mainly using animal  
82 models or *in vitro* systems <sup>10</sup>. Conversely, the molecular mechanisms controlling the  
83 disappearance of ghrelin from the circulation have been less investigated. Ghrelin rapidly  
84 disappears from plasma, having a half-life of 9-13 min after a single bolus injection of the  
85 hormone <sup>11-13</sup>, and the key chemical reactions affecting plasma ghrelin involve not only des-  
86 acylation but also proteolysis. Ghrelin des-acylation in plasma results in the generation of  
87 desacyl-ghrelin (DAG), which is also secreted from ghrelin cells. DAG does not bind to  
88 GHSR at physiological levels, but it has been suggested to display some GHSR-  
89 independent effects on insulin secretion, osteoblast growth and lipid metabolism <sup>1</sup>. Ghrelin  
90 des-acylation in plasma is presumably mediated by different esterases including platelet  
91 activating factor acetylhydrolase, paraoxonase,  $\alpha$ 2-macroglobulin, butyrylcholinesterase

92 and acyl protein thioesterase 1; although it is likely that other esterases can also hydrolyze  
93 the lipid moiety from ghrelin <sup>14-19</sup>. Ghrelin also undergoes proteolysis in plasma giving rise to  
94 shorter peptides <sup>15,16</sup>. Ghrelin proteolysis mainly occurs at the middle segment of the peptide  
95 (i.e. between Arg15 and Lys16) and generates peptides of different length, including  
96 peptides containing the N-terminal end of ghrelin that may be able to evoke some activation  
97 of GHSR although less potently than full-length ghrelin <sup>16,20,21</sup>. Ghrelin proteolysis was  
98 suggested to involve endopeptidase activated protein C in bovine plasma <sup>22</sup>, and insulin-  
99 degrading enzyme in an *in vitro* system <sup>23</sup>. Still, the molecular identity of the enzymatic  
100 system mediating ghrelin inactivation in plasma remains to be fully clarified.

101

102 Since its discovery, manipulating ghrelin system emerged as a putative strategy in  
103 the treatment of obesity and related diseases. In order to further reduce ghrelin actions in  
104 obesity and achieve additional beneficial effects, GHSR antagonists, ghrelin vaccines and  
105 GOAT inhibitors have been developed; however, none of these strategies reached the  
106 desired effects in human trials <sup>24</sup>. Notably, the inhibition of the extracellular inactivation of  
107 incretins has proved to be a critical strategy to improve glycaemic control in patients suffering  
108 type 2 diabetes <sup>25</sup>. In this context, understanding the molecular mechanisms controlling the  
109 level of ghrelin in plasma emerges as a fundamental step forward to not only better  
110 understand the biology of ghrelin but also find new pharmacological strategies to manipulate  
111 such levels and achieve beneficial effects. To the best of our knowledge, the extent to which  
112 des-acylation or proteolysis of ghrelin in plasma are affected by the daily feeding status or  
113 obesity has not been previously investigated. Here, we hypothesized that decrease of  
114 plasma ghrelin in individuals with obesity and/or in the postprandial state could be due to an  
115 increase in its extracellular processing. In order to test our hypothesis, we performed a single  
116 test meal study in individuals with either normal weight (NW) or with overweight or obesity  
117 (OW/OB) and assessed not only levels of ghrelin and DAG in plasma but also the specific  
118 levels of ghrelin des-acylation and ghrelin proteolysis in plasma.

## 119 **Materials and Methods**

120

121 2.1 Study design. The study was approved by the Institutional Ethical Committee of  
122 San Martin Hospital, La Plata (Protocol ID HSMLP2020/0044) and conducted according to  
123 the Declaration of Helsinki guidelines and Argentinian legal provisions governing clinical  
124 research in humans. Written informed consents were obtained from all participants.  
125 Participants were recruited via advertising in social media from August to October 2021.

126

127 2.2 Study participants. This intervention cross-sectional study included 20–45 years-  
128 old adults, without chronic medical illness, personal history of diabetes, history of medical  
129 condition, or medication related to obesity or diabetes risk status. The study included 21  
130 males and 19 females. Pregnant women were excluded from the study. Sample size was  
131 calculated to unmask differences in plasma ghrelin levels, assuming a standard deviation of  
132 3.26 fmol/ml<sup>26</sup>, 80% statistical power and 5% significance level in preprandial ghrelin level,  
133 leading to a minimal sample size of 40 participants (20 with NW and 20 with OW/OB).

134

135 2.3 Study protocol. All participants arrived at the facilities of the Institute in the  
136 morning after a 12-h overnight fast and underwent an initial evaluation, including a medical  
137 and family history. Weight and body composition were measured with the participants  
138 barefoot and wearing minimal clothing, on a bioimpedance scale (Omron HBF514,  
139 Argentina) with a resolution of 10 g. Height was measured with a Harpenden-type wall-  
140 mounted stadiometer (Holtain Ltd., United Kingdom) with a resolution of 1 mm. BMI was  
141 calculated by dividing weight by height squared (kg/m<sup>2</sup>). As described by the World Health  
142 Organization, participants were classified as NW when BMI was between 18.5 and 24.99  
143 Kg/m<sup>2</sup> or as OW/OB when BMI≥25 Kg/m<sup>2</sup><sup>27</sup>. Next, a preprandial blood sample was obtained.  
144 Participants were given a test meal (breakfast) that provided 20% of the individual's daily  
145 energy requirement as estimated using the "Body Weight Planner" digital application of the  
146 National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, USA)<sup>28</sup>. The  
147 breakfast composition was 56 kcal% carbohydrates, 12 kcal% protein and 34 kcal% lipids  
148 and comprised yogurt, cereals and almonds. Participants were asked to consume the entire  
149 breakfast in less than 15 min. Blood sampling was repeated at 60 min after starting breakfast  
150 (postprandial), when plasma ghrelin level is significantly reduced, according to a meta-  
151 analysis study<sup>5</sup>. Blood samples were collected in tubes containing either heparin, to assess  
152 glucose and insulin, ghrelin proteolysis and ghrelin des-acylation, or EDTA (1 mg/mL final)  
153 and p-hydroxy-mercuribenzoic acid (0.4 mM final), to assess ghrelin and DAG. For ghrelin

154 and DAG assays, plasma was immediately acidified with HCl (0.1 N final) <sup>29</sup>. All samples  
155 were stored frozen at -80°C until analysis.

156

157 2.4 Biochemical analyses. Glycemia was measured using an enzymatic procedure  
158 in a CM 250 (Wiener Lab, Argentina). Insulin level was assessed using chemiluminescence  
159 (Advia Centaur XP, Siemens, Germany). Insulin resistance was estimated by the  
160 homeostasis model assessment for insulin resistance (HOMA index) <sup>30</sup>. Ghrelin and DAG  
161 were assessed using specific enzyme immunoassays from Bertin Bioreagent (A05306 and  
162 DAG, respectively), and following manufacturer's instructions. Ghrelin/DAG ratio was  
163 computed on a molar basis as ghrelin concentration divided by DAG concentration. The food  
164 intake-induced change of plasma ghrelin was calculated as preprandial minus postprandial  
165 ghrelin concentration in molar basis.

166

167 2.5 Assessment of ghrelin des-acylation in plasma. Here, we used a biotinylated  
168 analogue of ghrelin consisting in the first 11 residues of ghrelin with the octanoyl moiety  
169 linked to Ser3 and a biotin attached to the ε-amino group of Arg (biotin-ghrelin or  
170 GSS(Oct)FLSPEHQR-biotin). To assess ghrelin des-acylation in plasma (Fig. 1A), biotin-  
171 ghrelin (0.01 µg/µL) was incubated with each plasma sample (20 % v/v) in phosphate buffer  
172 saline at 37 °C. After 60 min, trifluoroacetic acid (20 % final) was added to the tube to stop  
173 the reaction. We next used a reverse-phase chromatographic procedure to separate biotin-  
174 ghrelin from biotin-DAG, based on a previously described protocol <sup>2</sup>. Briefly, reaction  
175 mixtures for each plasma sample were applied to a ZipTip with C18 resin, according to  
176 manufacturer's instructions, and eluted with a 20% and 100%-CH<sub>3</sub>CN solutions in 0.1% TFA  
177 in order to elute biotin-DAG and biotin-ghrelin, respectively. After CH<sub>3</sub>CN evaporation, eluted  
178 samples were subjected to dot blot using PVDF membranes. Next, blot membranes were  
179 incubated with Vectastain Elite ABC kit (Vector Laboratories, cat # PK6200) for 2 h,  
180 according to manufacturer's protocols, and the presence of biotin-ghrelin and biotin-DAG  
181 was visualized using a 3-3'-diaminobenzidine (DAB)/Nickel solution, which gives a  
182 black/purple precipitate. DAB signal per spot was registered using a Gel Doc XR (Bio-Rad)  
183 and quantified using the software Fiji. Ghrelin des-acylation was calculated as the ratio  
184 between the intensity of biotin-DAG and biotin-ghrelin and expressed as relative units (r.u.).  
185 The accuracy of the separation and quantification system was checked using solutions with  
186 different concentrations of either intact biotin-ghrelin and biotin-DAG.

187

188            2.6 Assessment of ghrelin proteolysis in plasma. Here, we used a fluorescent  
189 analogue of human ghrelin (F-ghrelin) consisting in the first 19 residues of ghrelin with the  
190 octanoyl moiety linked to a diaminopropanoic acid (Dpr) at position 3 and a fluorescein  
191 isothiocyanate attached to the  $\epsilon$ -amino group of Lys19  
192 (GSDpr(Oct)FLSPEHQRVQQRKESK(FITC)-NH<sub>2</sub>), which was synthesized as previously  
193 described<sup>31</sup>. To assess ghrelin proteolysis in plasma (Fig. 1B), F-ghrelin (0.025  $\mu$ g/ $\mu$ L) was  
194 incubated with each plasma sample (12.5 % v/v) in phosphate buffer saline at 37 °C. After  
195 180 min, loading buffer was added to the tubes and reaction mixtures for each sample were  
196 loaded onto 20% polyacrylamide gels prepared according to the Ornstein-Davis  
197 discontinuous system. Fluorescence was visualized by trans-UV exposure, registered using  
198 a 520DF30 emission filter (Gel Doc XR, Bio-Rad) and quantified using the software Fiji.  
199 Ghrelin proteolysis was calculated as the ratio between the fluorescence of the shorter  
200 peptide products of cleavage and the fluorescence of intact F-ghrelin, and expressed as  
201 relative units (r.u.).

202

203            2.7 Statistics and data analyses. Statistical analyses were performed using  
204 GraphPad Prism 9.0.0 Normal distribution of data was tested using the Shapiro-Wilk test.  
205 Preprandial characteristics in males were compared with Student's unpaired t test or Mann  
206 Whitney test. Positively skewed variables (ghrelin, DAG, ghrelin/DAG, and ghrelin  
207 proteolysis) were log-transformed for all subsequent analyses. Changes in time between  
208 groups were evaluated with two-way repeated measures analysis of variance (ANOVA), with  
209 group (NW vs. OW/OB) as between factor and feeding condition (preprandial vs.  
210 postprandial) as within factor. Linear correlations between log-transformed ghrelin  
211 proteolysis and other variables were assessed using Pearson's (ghrelin, BMI and glycemia)  
212 or Spearman's (DAG) correlation coefficients. Tests used for each comparison were  
213 indicated in each figure legends. Variables with normal distribution were expressed as the  
214 mean  $\pm$  standard deviation (SD), and variables with non-normal distribution were reported  
215 as median (interquartile range [IQR]). Differences were considered statistically significant  
216 when  $p < 0.05$ .



## 217 Results

218

219 3.1 Ghrelin level decreases whereas ghrelin proteolysis increases in preprandial  
220 plasma of males with OW/OB. Initially, male participants were grouped as NW (n=9) or  
221 OW/OB (n=12). As shown in Table 1, age did not differ between groups nor correlate with  
222 any assessed parameter, whereas BMI, preprandial glycemia, preprandial insulin levels and  
223 HOMA index were significantly higher in OW/OB group, as compared to NW group.  
224 Conversely, preprandial ghrelin (Fig. 2A) and DAG (Fig. 2B) levels were ~2.2- and ~6.0-fold  
225 lower, respectively, in OW/OB group, as compared to NW group. Ghrelin/DAG ratio (Fig.  
226 2C) was ~8.5-fold higher in OW/OB group, as compared to NW group, suggesting that the  
227 mechanisms controlling plasma ghrelin level are differentially affected in males with OW/OB.  
228 Thus, we assessed the capacity of plasma samples to des-acylate a biotinylated ghrelin  
229 analogue. We found that ghrelin des-acylation was not different between plasma samples  
230 of NW and OW/OB groups (Fig. 2D). Next, we assessed the capacity of plasma samples to  
231 proteolyze a fluorescent ghrelin analogue. Here, ghrelin proteolysis was ~4.6-fold higher in  
232 OW/OB group, as compared to NW group (Fig. 2E). Notably, ghrelin proteolysis negatively  
233 correlated with plasma levels of ghrelin and DAG (Fig. 3A-B), it did not correlate with age  
234 ( $r=0.223$ ,  $p=0.3317$ , not shown), plasma insulin levels ( $r=0.269$ ,  $p=0.239$ , not shown), HOMA  
235 index ( $r=0.303$ ,  $p=0.182$ , not shown) nor BMI (Fig. 3C) and it positively correlated with  
236 glycemia (Fig. 3D).

237

238 3.2 Postprandial ghrelin proteolysis in plasma increases in males with NW, but not  
239 with OW/OB, whereas postprandial ghrelin des-acylation remains unaffected. After the  
240 single test meal intervention, glycemia was not affected by feeding condition ( $p$  condition=  
241 0.820), and remained higher in OW/OB group, as compared to NW group ( $p$  group=0.012).  
242 Plasma levels of ghrelin and DAG changed in a group- and feeding condition-dependent  
243 manner, being decreased in OW/OB group and postprandially (Fig. 4A-B). Here, the food  
244 intake-induced change of plasma ghrelin level tended to be lower in OW/OB group, as  
245 compared to NW group ( $6.5\pm 5.7$  vs.  $10.9\pm 7.8$  fmol/mL, respectively,  $P=0.0765$ ). Notably,  
246 ghrelin des-acylation remained unaffected by group or feeding condition (Fig. 4C). Ghrelin  
247 proteolysis also showed a significant interaction between the effects of group and feeding  
248 condition, being ~1.6-fold increased after food intake only in NW group and increased in  
249 OW/OB group, independently of the feeding condition (Fig. 4D).

250

251 3.3 Postprandial ghrelin proteolysis increases in NW females but remains unaffected

252 in plasma of females with OW/OB, independently of the feeding condition. Since some  
253 evidence indicate that ghrelin level fluctuates in a sex-dependent manner in humans <sup>32,33</sup>,  
254 we also studied females under the same study protocol and grouped them as NW (N=11)  
255 or OW/OB (n=8). As shown in Table 2, age and preprandial glycemia did not differ between  
256 groups whereas BMI, preprandial insulin levels and HOMA index were significantly higher  
257 in OW/OB group, as compared to NW group. In preprandial condition, ghrelin and DAG  
258 levels were ~2.0- and ~2.6-fold lower, respectively, in OW/OB group, but ghrelin/DAG ratio  
259 was not different between both groups, suggesting that the mechanisms controlling plasma  
260 ghrelin level in females with OW/OB were less affected than in males with OW/OB. Here,  
261 ghrelin des-acylation and ghrelin proteolysis in plasma were not different between NW and  
262 OW/OB groups (Table 2). In contrast to males, ghrelin proteolytic activity in plasma did not  
263 correlate with glycemia in females ( $r=0.154$ ,  $p=0.530$ ).

264  
265 After the test meal intervention, plasma levels of ghrelin and DAG changed in a  
266 group- and feeding condition-dependent manner, being decreased in OW/OB group and  
267 postprandially (Fig. 5A-B). Here, the food intake-induced change of plasma ghrelin level was  
268 lower in the OW/OB group, as compared to NW group ( $10.2\pm 8.3$  vs.  $26.4\pm 16.75$  fmol/mL,  
269 respectively,  $P=0.0230$ ). As found in males, ghrelin des-acylation was unaffected by the  
270 feeding condition in NW and OW/OB groups (not shown) whereas ghrelin proteolysis was  
271 postprandially increased ~2.2 folds only in NW group (Fig. 5C).

## 272 Discussion

273  
274 Here, we performed a single test meal study in individuals with NW or OW/OB and  
275 investigated levels of ghrelin, ghrelin des-acylation and ghrelin proteolysis in plasma. We  
276 confirmed that preprandial ghrelin level is lower in individuals with OW/OB, independently  
277 of sex, as previously shown <sup>5,8,9,32,34-38</sup>. After food intake, ghrelin level decreased in males  
278 and females of NW and OW/OB groups. Interestingly, ghrelin proteolysis was higher in  
279 plasma of males, but not females, with OW/OB and correlated with glycemia. Ghrelin  
280 proteolysis increased after food intake in individuals with NW only. In contrast, ghrelin des-  
281 acylation was similar in plasma of individuals with NW or OW/OB and remained unchanged  
282 after food intake.

283  
284 The current study was carefully designed to assess the chemical reactions affecting  
285 plasma ghrelin at two key time points: in the morning after overnight fasting, when plasma  
286 ghrelin is around the highest diurnal level <sup>8</sup>, and 60 min after meal, when plasma ghrelin  
287 decreases <sup>5</sup>. Indeed, we detected the expected postprandial decline in ghrelin level in most  
288 lean individuals (19 out of 20), as shown in several previous reports <sup>5,32,35-38</sup>, confirming that  
289 plasma ghrelin level was studied when it undergoes a significant change. It is important to  
290 highlight that it is now well established that the inaccurate manipulation of plasma samples  
291 results in the conversion of most ghrelin into DAG and a consequent low ghrelin/DAG ratio  
292 <sup>39</sup>. Here, plasma ghrelin/DAG ratio in NW individuals was ~0.6, which is similar to the highest  
293 reported ghrelin/DAG ratios in similar studies (see for instance <sup>40</sup>), indicating that the  
294 conditions of sampling and storage were accurate to preserve ghrelin in plasma despite the  
295 complexity involved in clinical studies. Finally, current study was performed in relatively  
296 young individuals in order to minimize the putative impact of the age-dependent decline in  
297 plasma ghrelin that has been observed older individuals <sup>41</sup>.

298  
299 In order to specifically assess ghrelin des-acylation or proteolysis in plasma, we set  
300 up two reproducible assays whose characteristics should be taken in consideration when  
301 interpreting current findings. Importantly, we used two different labelled variants of ghrelin  
302 that rely on modifications at its C-terminal end, which is not essential for bioactivity <sup>20,42</sup>. To  
303 assess ghrelin des-acylation, we used biotin-ghrelin, a 11-residue analogue of ghrelin  
304 biotinylated at the Arg11 C-terminus. Biotin-ghrelin is susceptible to hydrolysis by esterases,  
305 since the octanoyl moiety is coupled at Ser3 through an ester bond, but presumably resistant  
306 to plasma proteases, since ghrelin1-11 is the shorter derivative of ghrelin found after

307 proteolysis of full-length ghrelin<sup>22</sup>. To assess ghrelin proteolysis, we used F-ghrelin, an 18-  
308 residue analog of ghrelin conjugated to fluorescein isothiocyanate through a Lys added at  
309 its C terminus. F-ghrelin contains the entire peptide segment susceptible to proteolysis,  
310 according to previous descriptions<sup>22</sup>, but is resistant to esterases because Ser3 was  
311 replaced with Dpr, which is coupled with the octanoyl moiety through an amide bond<sup>31</sup>. Of  
312 note, F-ghrelin and the fluorescent products of proteolysis were directly visualized and  
313 quantified after separation by native gel electrophoresis. In contrast, visualization and  
314 quantification of biotin-ghrelin and biotin-DAG required several steps including reverse-  
315 phase chromatographic procedure for separation, dot-blot and chromogenic staining. Thus,  
316 it is possible that the method used to assess ghrelin des-acylation lacked enough sensitivity  
317 to unmask small differences in enzymatic activity. Also, reaction times to estimate each  
318 enzymatic activity were set up in order to have detectable amounts of substrate and products  
319 of each assay. Thus, ghrelin des-acylation and ghrelin proteolysis assays required 60 and  
320 180 min of reaction time, respectively. The observation that the hydrolysis of peptide bonds  
321 of ghrelin in plasma is a relatively slow process would suggest that changes in ghrelin  
322 proteolysis have stronger implications in long-term conditions, as we observed here in males  
323 with OW/OB. In contrast, the physiological implications of the detected postprandial increase  
324 of ghrelin proteolysis in plasma of lean individuals is uncertain given the difference between  
325 the timing for the rapid postprandial decrease of plasma ghrelin and the reaction time  
326 required to assess ghrelin proteolysis in our experimental conditions.

327

328 To the best of our knowledge, the current study provides the first indication that  
329 proteolysis of ghrelin in plasma increases in males with OW/OB. The observation that ghrelin  
330 proteolysis negatively correlates with plasma levels of ghrelin and DAG in the preprandial  
331 state suggests that increased ghrelin proteolysis in males with OW/OB may contribute to  
332 reduce plasma levels of these peptides. Also, increased ghrelin proteolysis in obesity may  
333 contribute to impair the effects of ghrelin treatment (the so-called ghrelin resistance),  
334 reported in obese mice<sup>43</sup>. Strikingly, we found here that ghrelin proteolysis positively  
335 correlated with glycemia, but did not correlate with insulinaemia or HOMA index. Conversely,  
336 preprandial ghrelin proteolysis and glycemia remained unaffected in females with OW/OB,  
337 despite they showed reduced preprandial plasma ghrelin level and insulin resistance, as  
338 compared to females with NW. Thus, preprandial ghrelin proteolysis is somehow associated  
339 to glucose level itself, via mechanisms that do not seem to involve insulin. The reasons why  
340 ghrelin proteolytic activity increases only in males with OW/OB are uncertain, but it is in line  
341 with the observation that ghrelin level in obesity tends to be lower in males than females, as

342 found here and reported in previous studies <sup>32,33</sup>. Interestingly, insulin-degrading enzyme  
343 level in plasma was found higher in individuals (of undisclosed sex) with metabolic syndrome  
344 and strongly correlated with the severity of the symptoms <sup>44</sup>. Also, plasma activated protein  
345 C level was higher in individuals (male and female combined) with obesity and reduced after  
346 bariatric surgery <sup>45,46</sup>. Thus, some proteases able to cleave ghrelin are strong candidates to  
347 mediate increased ghrelin proteolysis in plasma of male individuals with OW/OB.

348

349 Current study also provides the first indication that ghrelin proteolysis increases in  
350 plasma after food intake in lean individuals, in a sex-independent manner, allowing to  
351 hypothesize that increased postprandial ghrelin proteolysis in lean individuals contributes to  
352 decrease plasma ghrelin levels after food intake. Since glycemia is well-known to transiently  
353 increase after food intake, it is possible that the postprandial increment of ghrelin proteolysis  
354 is also linked to glucose levels. In line with this possibility, a previous study found that a  
355 glucose load potentially decreases ghrelin levels in lean individuals <sup>31</sup>. Further studies  
356 assessing blood samples at earlier time points will be required to test the extent to which  
357 postprandial increments of glycemia and ghrelin proteolysis correlate. Interestingly, ghrelin  
358 proteolysis did not increase after food intake in plasma of individuals with OW/OB, in which  
359 plasma ghrelin level shows a smaller decrease after meal intake, as previously shown <sup>5</sup> and  
360 confirmed here. Thus, it is likely that the lack of postprandial increase in ghrelin proteolysis  
361 in obesity contributes to impair the postprandial reduction of ghrelin levels, an alteration that  
362 has been proposed to reduce satiety feelings in individuals with obesity <sup>47-49</sup>.

363

364 Although informative, this study is not without limitations. For instance, some aspects  
365 of our experimental design, such as the postprandial blood sample collection time or the  
366 sample size, were based on previous studies investigating the postprandial decline in ghrelin  
367 level <sup>5,32,35-38</sup>. However, such experimental design may have prevented us from detecting the  
368 postprandial change of ghrelin proteolytic activity in individuals with OW/OB, if an increase  
369 in such enzymatic activity takes place with a different kinetics or if it requires a higher sample  
370 size to be unmasked. Also, the time chosen to collect the postprandial blood sample did not  
371 allow us to detect the transient meal-induced increase of glycemia and, consequently, we  
372 could not evaluate if postprandial hyperglycaemia either was differentially affected in  
373 individuals with OW/OB or correlated the postprandial increase of ghrelin proteolytic activity  
374 in lean individuals. The study did not include “sham-feeding” control groups and,  
375 consequently, a postprandial increase of ghrelin proteolytic activity may be related to its

376 physiological diurnal rhythm. Despite these limitations, the current study was very valuable  
377 to gain insights about a novel aspect of the biology of ghrelin in human plasma.

378

379 Ghrelin des-acylation in plasma is fast and affects the circulating ghrelin level <sup>50</sup>.  
380 Indeed, intravenous administration of ghrelin in humans results in a peak of ghrelin levels,  
381 at 15 min after injection, and is rapidly eliminated <sup>11,40</sup>. Ghrelin des-acylation seems to be  
382 the main mechanism mediating the reduction of plasma ghrelin elevation since the referred  
383 increase of ghrelin level is followed by a peak of DAG level, which then disappears from  
384 plasma more slowly than ghrelin <sup>11,40</sup>. Here, we found that ghrelin des-acylation was  
385 unaffected in individuals with OW/OB or postprandially suggesting that des-acylation is not  
386 a key mechanism mediating the decrease of plasma ghrelin level in those metabolic  
387 conditions. In line with our observations, it was reported that the level of serum  
388 butyrylcholinesterase activity, a ghrelin esterase in humans, was not altered during fasting  
389 <sup>51</sup>. Since our results indicate that ghrelin proteolysis is an important mechanism affecting the  
390 decrease of plasma ghrelin level in males with OW/OB, the mechanisms mediating the  
391 decrease of plasma ghrelin level in females with OW/OB remain uncertain. Given the fast  
392 rate of ghrelin des-acylation, it can be hypothesized that the observed decrease of plasma  
393 ghrelin level in the above referred conditions is due to a reduction of the secretion of ghrelin  
394 from the stomach <sup>10</sup>, as it has been shown in obese mice <sup>52</sup>. Also, it is possible that des-  
395 acylation of circulating ghrelin is unaffected in plasma but regulated in some specific tissues,  
396 such as liver. Future studies in humans are needed to test these possibilities.

397

## 398 **Conclusions**

399 We provide here evidence indicating that ghrelin proteolysis in plasma increases in  
400 males with OW/OB and that it is acutely regulated by food intake in lean individuals. Also,  
401 we report that ghrelin des-acylation in plasma is not affected by body weight or food intake.  
402 Thus, current study helps to better understand the mechanisms controlling the changes of  
403 plasma ghrelin under different metabolic conditions in humans. Ghrelin proteolysis may be  
404 a putative pharmacological target to control circulating ghrelin level in humans. In particular,  
405 enhancement of ghrelin proteolysis in males with OW/OB or after food intake may help to  
406 further reduce plasma ghrelin level, with concomitant “beneficial” effects such as appetite  
407 reduction and downregulation of mechanisms that increase glycemia.

408

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418

419 **Conflict of interest**

420 The authors declare no conflicts of interest. The sponsors had no role in the design of the  
421 study, the collection, analyses or interpretation of data, writing of the manuscript, or the  
422 decision to publish the results.

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581

582 **Figure Legends.**

583

584 Figure 1. Overview of methods to assess ghrelin des-acylation (A) and ghrelin proteolysis  
585 (B). See text for details. Eppendorf tube illustration adapted from scidraw.io  
586 (doi.org/10.5281/zenodo.6808872).

587

588 Figure 2. Preprandial plasma levels of ghrelin (A), DAG (B), ghrelin/DAG ratio (C), ghrelin  
589 des-acylation activity (D) and ghrelin proteolysis (E) in NW and OW/OB male participants.  
590 \*, \*\*, \*\*\* and \*\*\*\* indicate significant difference at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.0001$  by  
591 Student's t test or Mann Whitney test.

592

593 Figure 3. Correlations of ghrelin proteolysis with ghrelin (A), DAG (B), BMI (C), and glycemia  
594 (D) in male participants in preprandial conditions.

595

596 Figure 4. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B), ghrelin des-  
597 acylation activity (C) and ghrelin proteolysis (D) in males with NW or OW/OB. Coloured  
598 points and lines show the geometric mean and each grey line represents the change within  
599 an individual participant. Exact P values are shown. Two-way repeated measures analysis  
600 of variance (ANOVA) with Bonferroni multiple-comparisons test was performed. \*: significant  
601 difference vs. same group but different feeding condition. #: significant difference vs.  
602 different group but same feeding condition.

603

604 Figure 5. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B) and ghrelin  
605 proteolysis (C) in **females** with NW or OW/OB. Coloured points and lines show the geometric  
606 mean and each grey line represents the change within an individual participant. Exact P  
607 values are shown. Two-way repeated measures analysis of variance (ANOVA) with  
608 Bonferroni multiple-comparisons test was performed. \*: significant difference vs. same group  
609 but different feeding condition. #: significant difference vs. different group but same feeding  
610 condition.

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## **Ghrelin Proteolysis Increases in Plasma of Men, but not Women, with Obesity**

**Running title:** ghrelin proteolysis in human obesity

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**Running title:** ghrelin proteolysis in human obesity

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34 **Abstract**

35

36 Aims: Since plasma ghrelin can undergo des-acylation and proteolysis, the aim of this study  
37 was to investigate the extent to which an enhancement of these reactions is associated to  
38 the decrease of ghrelin in plasma after food intake or in individuals with obesity.

39 Main methods: we performed an intervention cross-sectional study, in which levels of  
40 ghrelin, desacyl-ghrelin (DAG), glucose, insulin, ghrelin des-acylation and ghrelin  
41 proteolysis were assessed in plasma before and after a test meal in 40 people (n=21 males)  
42 with normal weight (NW, n=20) or overweight/obesity (OW/OB, n=20).

43 Key findings: Preprandial ghrelin and DAG levels were lower, whereas preprandial ghrelin  
44 proteolysis was ~4.6-fold higher in plasma of males with OW/OB. In males, ghrelin  
45 proteolysis positively correlated with glycemia. Ghrelin and DAG levels were also lower in  
46 females with OW/OB, but preprandial ghrelin proteolysis was not different between females  
47 with NW or OW/OB. Ghrelin and DAG levels decreased postprandially in males and females,  
48 independently of BMI, and ghrelin proteolysis increased postprandially ~2 folds only in  
49 individuals with NW. Ghrelin des-acylation remained unaffected by BMI or feeding status in  
50 both sexes.

51 Significance: Current study shows that ghrelin proteolysis increases in males with obesity  
52 as well as after meal in lean individuals. Therefore, ghrelin proteolysis may be an important  
53 checkpoint and, consequently, a putative pharmacological target to control circulating  
54 ghrelin levels in humans.

55

56 **Key words**: ghrelin proteolysis, ghrelin des-acylation, obesity.

## 57 Introduction

58

59 Ghrelin is a 28-residue peptide hormone predominantly secreted by cells of the  
60 stomach that displays several unique biochemical and biological features <sup>1</sup>. Chemically,  
61 ghrelin is the only peptide known to be acylated with an octanoic moiety, a posttranslational  
62 modification that takes place at Ser3 of ghrelin and is catalysed by the enzyme ghrelin-O-  
63 acyltransferase (GOAT) early in the biosynthetic processing within the ghrelin-producing  
64 cells <sup>2</sup>. The biological actions of ghrelin, which are mediated by the growth hormone  
65 secretagogue receptor (GHSR), entirely depend on the octanoylation of ghrelin and include  
66 the most potent orexigenic effect described to date <sup>3,4</sup>. Ghrelin also induces growth hormone  
67 and glucocorticoid secretion, recruits several mechanisms that increase glycemia, increases  
68 gastrointestinal motility and affects non-homeostatic aspects of food intake, among other  
69 effects <sup>1</sup>. Thus, ghrelin is a pleiotropic hormone that contains an exceptional  
70 posttranslational modification that is essential for its bioactivity.

71

72 Plasma ghrelin level is tightly regulated by the daily feeding status and by the long-  
73 term energy balance. In the short term, ghrelin level increases before meals and decreases  
74 to baseline levels within the first hour after meals <sup>5</sup>. Ghrelin level increases in prolonged  
75 energy deficit conditions, such as fasting, calorie restriction or anorexia nervosa <sup>6,7</sup>, when a  
76 rise in plasma ghrelin contributes to seek for food and maintain glycemia. In contrast, ghrelin  
77 level decreases under energy surplus conditions, such as obesity <sup>8,9</sup>, and such decrease  
78 presumably results as a compensatory adaptation aiming to reduce feeding and glycemia.  
79 As other hormones, plasma ghrelin level depends on the equilibrium among its secretion,  
80 degradation and clearance rates. A large body of studies characterized the mechanisms  
81 controlling the biosynthesis and release of ghrelin from ghrelin cells mainly using animal  
82 models or *in vitro* systems <sup>10</sup>. Conversely, the molecular mechanisms controlling the  
83 disappearance of ghrelin from the circulation have been less investigated. Ghrelin rapidly  
84 disappears from plasma, having a half-life of 9-13 min after a single bolus injection of the  
85 hormone <sup>11-13</sup>, and the key chemical reactions affecting plasma ghrelin involve not only des-  
86 acylation but also proteolysis. Ghrelin des-acylation in plasma results in the generation of  
87 desacyl-ghrelin (DAG), which is also secreted from ghrelin cells. DAG does not bind to  
88 GHSR at physiological levels, but it has been suggested to display some GHSR-  
89 independent effects on insulin secretion, osteoblast growth and lipid metabolism <sup>1</sup>. Ghrelin  
90 des-acylation in plasma is presumably mediated by different esterases including platelet  
91 activating factor acetylhydrolase, paraoxonase,  $\alpha$ 2-macroglobulin, butyrylcholinesterase

92 and acyl protein thioesterase 1; although it is likely that other esterases can also hydrolyze  
93 the lipid moiety from ghrelin <sup>14-19</sup>. Ghrelin also undergoes proteolysis in plasma giving rise to  
94 shorter peptides <sup>15,16</sup>. Ghrelin proteolysis mainly occurs at the middle segment of the peptide  
95 (i.e. between Arg15 and Lys16) and generates peptides of different length, including  
96 peptides containing the N-terminal end of ghrelin that may be able to evoke some activation  
97 of GHSR although less potently than full-length ghrelin <sup>16,20,21</sup>. Ghrelin proteolysis was  
98 suggested to involve endopeptidase activated protein C in bovine plasma <sup>22</sup>, and insulin-  
99 degrading enzyme in an *in vitro* system <sup>23</sup>. Still, the molecular identity of the enzymatic  
100 system mediating ghrelin inactivation in plasma remains to be fully clarified.

101

102         Since its discovery, manipulating ghrelin system emerged as a putative strategy in  
103 the treatment of obesity and related diseases. In order to further reduce ghrelin actions in  
104 obesity and achieve additional beneficial effects, GHSR antagonists, ghrelin vaccines and  
105 GOAT inhibitors have been developed; however, none of these strategies reached the  
106 desired effects in human trials <sup>24</sup>. Notably, the inhibition of the extracellular inactivation of  
107 incretins has proved to be a critical strategy to improve glycaemic control in patients suffering  
108 type 2 diabetes <sup>25</sup>. In this context, understanding the molecular mechanisms controlling the  
109 level of ghrelin in plasma emerges as a fundamental step forward to not only better  
110 understand the biology of ghrelin but also find new pharmacological strategies to manipulate  
111 such levels and achieve beneficial effects. To the best of our knowledge, the extent to which  
112 des-acylation or proteolysis of ghrelin in plasma are affected by the daily feeding status or  
113 obesity has not been previously investigated. Here, we hypothesized that decrease of  
114 plasma ghrelin in individuals with obesity and/or in the postprandial state could be due to an  
115 increase in its extracellular processing. In order to test our hypothesis, we performed a single  
116 test meal study in individuals with either normal weight (NW) or with overweight or obesity  
117 (OW/OB) and assessed not only levels of ghrelin and DAG in plasma but also the specific  
118 levels of ghrelin des-acylation and ghrelin proteolysis in plasma.

## 119 **Materials and Methods**

120

121 2.1 Study design. The study was approved by the Institutional Ethical Committee of  
122 San Martin Hospital, La Plata (Protocol ID HSMLP2020/0044) and conducted according to  
123 the Declaration of Helsinki guidelines and Argentinian legal provisions governing clinical  
124 research in humans. Written informed consents were obtained from all participants.  
125 Participants were recruited via advertising in social media from August to October 2021.

126

127 2.2 Study participants. This intervention cross-sectional study included 20–45 years-  
128 old adults, without chronic medical illness, personal history of diabetes, history of medical  
129 condition, or medication related to obesity or diabetes risk status. The study included 21  
130 males and 19 females. Pregnant women were excluded from the study. Sample size was  
131 calculated to unmask differences in plasma ghrelin levels, assuming a standard deviation of  
132 3.26 fmol/ml<sup>26</sup>, 80% statistical power and 5% significance level in preprandial ghrelin level,  
133 leading to a minimal sample size of 40 participants (20 with NW and 20 with OW/OB).

134

135 2.3 Study protocol. All participants arrived at the facilities of the Institute in the  
136 morning after a 12-h overnight fast and underwent an initial evaluation, including a medical  
137 and family history. Weight and body composition were measured with the participants  
138 barefoot and wearing minimal clothing, on a bioimpedance scale (Omron HBF514,  
139 Argentina) with a resolution of 10 g. Height was measured with a Harpenden-type wall-  
140 mounted stadiometer (Holtain Ltd., United Kingdom) with a resolution of 1 mm. BMI was  
141 calculated by dividing weight by height squared (kg/m<sup>2</sup>). As described by the World Health  
142 Organization, participants were classified as NW when BMI was between 18.5 and 24.99  
143 Kg/m<sup>2</sup> or as OW/OB when BMI $\geq$ 25 Kg/m<sup>2</sup><sup>27</sup>. Next, a preprandial blood sample was obtained.  
144 Participants were given a test meal (breakfast) that provided 20% of the individual's daily  
145 energy requirement as estimated using the "Body Weight Planner" digital application of the  
146 National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, USA)<sup>28</sup>. The  
147 breakfast composition was 56 kcal% carbohydrates, 12 kcal% protein and 34 kcal% lipids  
148 and comprised yogurt, cereals and almonds. Participants were asked to consume the entire  
149 breakfast in less than 15 min. Blood sampling was repeated at 60 min after starting breakfast  
150 (postprandial), when plasma ghrelin level is significantly reduced, according to a meta-  
151 analysis study<sup>5</sup>. Blood samples were collected in tubes containing either heparin, to assess  
152 glucose and insulin, ghrelin proteolysis and ghrelin des-acylation, or EDTA (1 mg/mL final)  
153 and p-hydroxy-mercuribenzoic acid (0.4 mM final), to assess ghrelin and DAG. For ghrelin

154 and DAG assays, plasma was immediately acidified with HCl (0.1 N final) <sup>29</sup>. All samples  
155 were stored frozen at -80°C until analysis.

156

157 2.4 Biochemical analyses. Glycemia was measured using an enzymatic procedure  
158 in a CM 250 (Wiener Lab, Argentina). Insulin level was assessed using chemiluminescence  
159 (Advia Centaur XP, Siemens, Germany). Insulin resistance was estimated by the  
160 homeostasis model assessment for insulin resistance (HOMA index) <sup>30</sup>. Ghrelin and DAG  
161 were assessed using specific enzyme immunoassays from Bertin Bioreagent (A05306 and  
162 DAG, respectively), and following manufacturer's instructions. Ghrelin/DAG ratio was  
163 computed on a molar basis as ghrelin concentration divided by DAG concentration. The food  
164 intake-induced change of plasma ghrelin was calculated as preprandial minus postprandial  
165 ghrelin concentration in molar basis.

166

167 2.5 Assessment of ghrelin des-acylation in plasma. Here, we used a biotinylated  
168 analogue of ghrelin consisting in the first 11 residues of ghrelin with the octanoyl moiety  
169 linked to Ser3 and a biotin attached to the ε-amino group of Arg (biotin-ghrelin or  
170 GSS(Oct)FLSPEHQR-biotin). To assess ghrelin des-acylation in plasma (Fig. 1A), biotin-  
171 ghrelin (0.01 µg/µL) was incubated with each plasma sample (20 % v/v) in phosphate buffer  
172 saline at 37 °C. After 60 min, trifluoroacetic acid (20 % final) was added to the tube to stop  
173 the reaction. We next used a reverse-phase chromatographic procedure to separate biotin-  
174 ghrelin from biotin-DAG, based on a previously described protocol <sup>2</sup>. Briefly, reaction  
175 mixtures for each plasma sample were applied to a ZipTip with C18 resin, according to  
176 manufacturer's instructions, and eluted with a 20% and 100%-CH<sub>3</sub>CN solutions in 0.1% TFA  
177 in order to elute biotin-DAG and biotin-ghrelin, respectively. After CH<sub>3</sub>CN evaporation, eluted  
178 samples were subjected to dot blot using PVDF membranes. Next, blot membranes were  
179 incubated with Vectastain Elite ABC kit (Vector Laboratories, cat # PK6200) for 2 h,  
180 according to manufacturer's protocols, and the presence of biotin-ghrelin and biotin-DAG  
181 was visualized using a 3-3'-diaminobenzidine (DAB)/Nickel solution, which gives a  
182 black/purple precipitate. DAB signal per spot was registered using a Gel Doc XR (Bio-Rad)  
183 and quantified using the software Fiji. Ghrelin des-acylation was calculated as the ratio  
184 between the intensity of biotin-DAG and biotin-ghrelin and expressed as relative units (r.u.).  
185 The accuracy of the separation and quantification system was checked using solutions with  
186 different concentrations of either intact biotin-ghrelin and biotin-DAG.

187

188            2.6 Assessment of ghrelin proteolysis in plasma. Here, we used a fluorescent  
189 analogue of human ghrelin (F-ghrelin) consisting in the first 19 residues of ghrelin with the  
190 octanoyl moiety linked to a diaminopropanoic acid (Dpr) at position 3 and a fluorescein  
191 isothiocyanate attached to the  $\epsilon$ -amino group of Lys19  
192 (GSDpr(Oct)FLSPEHQRVQQRKESK(FITC)-NH<sub>2</sub>), which was synthesized as previously  
193 described<sup>31</sup>. To assess ghrelin proteolysis in plasma (Fig. 1B), F-ghrelin (0.025  $\mu$ g/ $\mu$ L) was  
194 incubated with each plasma sample (12.5 % v/v) in phosphate buffer saline at 37 °C. After  
195 180 min, loading buffer was added to the tubes and reaction mixtures for each sample were  
196 loaded onto 20% polyacrylamide gels prepared according to the Ornstein-Davis  
197 discontinuous system. Fluorescence was visualized by trans-UV exposure, registered using  
198 a 520DF30 emission filter (Gel Doc XR, Bio-Rad) and quantified using the software Fiji.  
199 Ghrelin proteolysis was calculated as the ratio between the fluorescence of the shorter  
200 peptide products of cleavage and the fluorescence of intact F-ghrelin, and expressed as  
201 relative units (r.u.).

202

203            2.7 Statistics and data analyses. Statistical analyses were performed using  
204 GraphPad Prism 9.0.0 Normal distribution of data was tested using the Shapiro-Wilk test.  
205 Preprandial characteristics in males were compared with Student's unpaired t test or Mann  
206 Whitney test. Positively skewed variables (ghrelin, DAG, ghrelin/DAG, and ghrelin  
207 proteolysis) were log-transformed for all subsequent analyses. Changes in time between  
208 groups were evaluated with two-way repeated measures analysis of variance (ANOVA), with  
209 group (NW vs. OW/OB) as between factor and feeding condition (preprandial vs.  
210 postprandial) as within factor. Linear correlations between log-transformed ghrelin  
211 proteolysis and other variables were assessed using Pearson's (ghrelin, BMI and glycemia)  
212 or Spearman's (DAG) correlation coefficients. Tests used for each comparison were  
213 indicated in each figure legends. Variables with normal distribution were expressed as the  
214 mean  $\pm$  standard deviation (SD), and variables with non-normal distribution were reported  
215 as median (interquartile range [IQR]). Differences were considered statistically significant  
216 when  $p < 0.05$ .

## 217 Results

218

219 3.1 Ghrelin level decreases whereas ghrelin proteolysis increases in preprandial  
220 plasma of males with OW/OB. Initially, male participants were grouped as NW (n=9) or  
221 OW/OB (n=12). As shown in Table 1, age did not differ between groups nor correlate with  
222 any assessed parameter, whereas BMI, preprandial glycemia, preprandial insulin levels and  
223 HOMA index were significantly higher in OW/OB group, as compared to NW group.  
224 Conversely, preprandial ghrelin (Fig. 2A) and DAG (Fig. 2B) levels were ~2.2- and ~6.0-fold  
225 lower, respectively, in OW/OB group, as compared to NW group. Ghrelin/DAG ratio (Fig.  
226 2C) was ~8.5-fold higher in OW/OB group, as compared to NW group, suggesting that the  
227 mechanisms controlling plasma ghrelin level are differentially affected in males with OW/OB.  
228 Thus, we assessed the capacity of plasma samples to des-acylate a biotinylated ghrelin  
229 analogue. We found that ghrelin des-acylation was not different between plasma samples  
230 of NW and OW/OB groups (Fig. 2D). Next, we assessed the capacity of plasma samples to  
231 proteolyze a fluorescent ghrelin analogue. Here, ghrelin proteolysis was ~4.6-fold higher in  
232 OW/OB group, as compared to NW group (Fig. 2E). Notably, ghrelin proteolysis negatively  
233 correlated with plasma levels of ghrelin and DAG (Fig. 3A-B), it did not correlate with age  
234 ( $r=0.223$ ,  $p=0.3317$ , not shown), plasma insulin levels ( $r=0.269$ ,  $p=0.239$ , not shown), HOMA  
235 index ( $r=0.303$ ,  $p=0.182$ , not shown) nor BMI (Fig. 3C) and it positively correlated with  
236 glycemia (Fig. 3D).

237

238 3.2 Postprandial ghrelin proteolysis in plasma increases in males with NW, but not  
239 with OW/OB, whereas postprandial ghrelin des-acylation remains unaffected. After the  
240 single test meal intervention, glycemia was not affected by feeding condition ( $p$  condition=  
241 0.820), and remained higher in OW/OB group, as compared to NW group ( $p$  group=0.012).  
242 Plasma levels of ghrelin and DAG changed in a group- and feeding condition-dependent  
243 manner, being decreased in OW/OB group and postprandially (Fig. 4A-B). Here, the food  
244 intake-induced change of plasma ghrelin level tended to be lower in OW/OB group, as  
245 compared to NW group ( $6.5\pm 5.7$  vs.  $10.9\pm 7.8$  fmol/mL, respectively,  $P=0.0765$ ). Notably,  
246 ghrelin des-acylation remained unaffected by group or feeding condition (Fig. 4C). Ghrelin  
247 proteolysis also showed a significant interaction between the effects of group and feeding  
248 condition, being ~1.6-fold increased after food intake only in NW group and increased in  
249 OW/OB group, independently of the feeding condition (Fig. 4D).

250

251 3.3 Postprandial ghrelin proteolysis increases in NW females but remains unaffected  
252 in plasma of females with OW/OB, independently of the feeding condition. Since some  
253 evidence indicate that ghrelin level fluctuates in a sex-dependent manner in humans <sup>32,33</sup>,  
254 we also studied females under the same study protocol and grouped them as NW (N=11)  
255 or OW/OB (n=8). As shown in Table 2, age and preprandial glycemia did not differ between  
256 groups whereas BMI, preprandial insulin levels and HOMA index were significantly higher  
257 in OW/OB group, as compared to NW group. In preprandial condition, ghrelin and DAG  
258 levels were ~2.0- and ~2.6-fold lower, respectively, in OW/OB group, but ghrelin/DAG ratio  
259 was not different between both groups, suggesting that the mechanisms controlling plasma  
260 ghrelin level in females with OW/OB were less affected than in males with OW/OB. Here,  
261 ghrelin des-acylation and ghrelin proteolysis in plasma were not different between NW and  
262 OW/OB groups (Table 2). In contrast to males, ghrelin proteolytic activity in plasma did not  
263 correlate with glycemia in females ( $r=0.154$ ,  $p=0.530$ ).

264

265 After the test meal intervention, plasma levels of ghrelin and DAG changed in a  
266 group- and feeding condition-dependent manner, being decreased in OW/OB group and  
267 postprandially (Fig. 5A-B). Here, the food intake-induced change of plasma ghrelin level was  
268 lower in the OW/OB group, as compared to NW group ( $10.2\pm 8.3$  vs.  $26.4\pm 16.75$  fmol/mL,  
269 respectively,  $P=0.0230$ ). As found in males, ghrelin des-acylation was unaffected by the  
270 feeding condition in NW and OW/OB groups (not shown) whereas ghrelin proteolysis was  
271 postprandially increased ~2.2 folds only in NW group (Fig. 5C).



272 **Discussion**

273

274 Here, we performed a single test meal study in individuals with NW or OW/OB and  
275 investigated levels of ghrelin, ghrelin des-acylation and ghrelin proteolysis in plasma. We  
276 confirmed that preprandial ghrelin level is lower in individuals with OW/OB, independently  
277 of sex, as previously shown <sup>5,8,9,32,34-38</sup>. After food intake, ghrelin level decreased in males  
278 and females of NW and OW/OB groups. Interestingly, ghrelin proteolysis was higher in  
279 plasma of males, but not females, with OW/OB and correlated with glycemia. Ghrelin  
280 proteolysis increased after food intake in individuals with NW only. In contrast, ghrelin des-  
281 acylation was similar in plasma of individuals with NW or OW/OB and remained unchanged  
282 after food intake.

283

284 The current study was carefully designed to assess the chemical reactions affecting  
285 plasma ghrelin at two key time points: in the morning after overnight fasting, when plasma  
286 ghrelin is around the highest diurnal level <sup>8</sup>, and 60 min after meal, when plasma ghrelin  
287 decreases <sup>5</sup>. Indeed, we detected the expected postprandial decline in ghrelin level in most  
288 lean individuals (19 out of 20), as shown in several previous reports <sup>5,32,35-38</sup>, confirming that  
289 plasma ghrelin level was studied when it undergoes a significant change. It is important to  
290 highlight that it is now well established that the inaccurate manipulation of plasma samples  
291 results in the conversion of most ghrelin into DAG and a consequent low ghrelin/DAG ratio  
292 <sup>39</sup>. Here, plasma ghrelin/DAG ratio in NW individuals was ~0.6, which is similar to the highest  
293 reported ghrelin/DAG ratios in similar studies (see for instance <sup>40</sup>), indicating that the  
294 conditions of sampling and storage were accurate to preserve ghrelin in plasma despite the  
295 complexity involved in clinical studies. Finally, current study was performed in relatively  
296 young individuals in order to minimize the putative impact of the age-dependent decline in  
297 plasma ghrelin that has been observed older individuals <sup>41</sup>.

298

299 In order to specifically assess ghrelin des-acylation or proteolysis in plasma, we set  
300 up two reproducible assays whose characteristics should be taken in consideration when  
301 interpreting current findings. Importantly, we used two different labelled variants of ghrelin  
302 that rely on modifications at its C-terminal end, which is not essential for bioactivity <sup>20,42</sup>. To  
303 assess ghrelin des-acylation, we used biotin-ghrelin, a 11-residue analogue of ghrelin  
304 biotinylated at the Arg11 C-terminus. Biotin-ghrelin is susceptible to hydrolysis by esterases,  
305 since the octanoyl moiety is coupled at Ser3 through an ester bond, but presumably resistant  
306 to plasma proteases, since ghrelin1-11 is the shorter derivative of ghrelin found after

307 proteolysis of full-length ghrelin <sup>22</sup>. To assess ghrelin proteolysis, we used F-ghrelin, an 18-  
308 residue analog of ghrelin conjugated to fluorescein isothiocyanate through a Lys added at  
309 its C terminus. F-ghrelin contains the entire peptide segment susceptible to proteolysis,  
310 according to previous descriptions <sup>22</sup>, but is resistant to esterases because Ser3 was  
311 replaced with Dpr, which is coupled with the octanoyl moiety through an amide bond <sup>31</sup>. Of  
312 note, F-ghrelin and the fluorescent products of proteolysis were directly visualized and  
313 quantified after separation by native gel electrophoresis. In contrast, visualization and  
314 quantification of biotin-ghrelin and biotin-DAG required several steps including reverse-  
315 phase chromatographic procedure for separation, dot-blot and chromogenic staining. Thus,  
316 it is possible that the method used to assess ghrelin des-acylation lacked enough sensitivity  
317 to unmask small differences in enzymatic activity. Also, reaction times to estimate each  
318 enzymatic activity were set up in order to have detectable amounts of substrate and products  
319 of each assay. Thus, ghrelin des-acylation and ghrelin proteolysis assays required 60 and  
320 180 min of reaction time, respectively. The observation that the hydrolysis of peptide bonds  
321 of ghrelin in plasma is a relatively slow process would suggest that changes in ghrelin  
322 proteolysis have stronger implications in long-term conditions, as we observed here in males  
323 with OW/OB. In contrast, the physiological implications of the detected postprandial increase  
324 of ghrelin proteolysis in plasma of lean individuals is uncertain given the difference between  
325 the timing for the rapid postprandial decrease of plasma ghrelin and the reaction time  
326 required to assess ghrelin proteolysis in our experimental conditions.

327

328 To the best of our knowledge, the current study provides the first indication that  
329 proteolysis of ghrelin in plasma increases in males with OW/OB. The observation that ghrelin  
330 proteolysis negatively correlates with plasma levels of ghrelin and DAG in the preprandial  
331 state suggests that increased ghrelin proteolysis in males with OW/OB may contribute to  
332 reduce plasma levels of these peptides. Also, increased ghrelin proteolysis in obesity may  
333 contribute to impair the effects of ghrelin treatment (the so-called ghrelin resistance),  
334 reported in obese mice <sup>43</sup>. Strikingly, we found here that ghrelin proteolysis positively  
335 correlated with glycemia, but did not correlate with insulinaemia or HOMA index. Conversely,  
336 preprandial ghrelin proteolysis and glycemia remained unaffected in females with OW/OB,  
337 despite they showed reduced preprandial plasma ghrelin level and insulin resistance, as  
338 compared to females with NW. Thus, preprandial ghrelin proteolysis is somehow associated  
339 to glucose level itself, via mechanisms that do not seem to involve insulin. The reasons why  
340 ghrelin proteolytic activity increases only in males with OW/OB are uncertain, but it is in line  
341 with the observation that ghrelin level in obesity tends to be lower in males than females, as

342 found here and reported in previous studies <sup>32,33</sup>. Interestingly, insulin-degrading enzyme  
343 level in plasma was found higher in individuals (of undisclosed sex) with metabolic syndrome  
344 and strongly correlated with the severity of the symptoms <sup>44</sup>. Also, plasma activated protein  
345 C level was higher in individuals (male and female combined) with obesity and reduced after  
346 bariatric surgery <sup>45,46</sup>. Thus, some proteases able to cleave ghrelin are strong candidates to  
347 mediate increased ghrelin proteolysis in plasma of male individuals with OW/OB.

348

349 Current study also provides the first indication that ghrelin proteolysis increases in  
350 plasma after food intake in lean individuals, in a sex-independent manner, allowing to  
351 hypothesize that increased postprandial ghrelin proteolysis in lean individuals contributes to  
352 decrease plasma ghrelin levels after food intake. Since glycemia is well-known to transiently  
353 increase after food intake, it is possible that the postprandial increment of ghrelin proteolysis  
354 is also linked to glucose levels. In line with this possibility, a previous study found that a  
355 glucose load potentially decreases ghrelin levels in lean individuals <sup>31</sup>. Further studies  
356 assessing blood samples at earlier time points will be required to test the extent to which  
357 postprandial increments of glycemia and ghrelin proteolysis correlate. Interestingly, ghrelin  
358 proteolysis did not increase after food intake in plasma of individuals with OW/OB, in which  
359 plasma ghrelin level shows a smaller decrease after meal intake, as previously shown <sup>5</sup> and  
360 confirmed here. Thus, it is likely that the lack of postprandial increase in ghrelin proteolysis  
361 in obesity contributes to impair the postprandial reduction of ghrelin levels, an alteration that  
362 has been proposed to reduce satiety feelings in individuals with obesity <sup>47-49</sup>.

363

364 Although informative, this study is not without limitations. For instance, some aspects  
365 of our experimental design, such as the postprandial blood sample collection time or the  
366 sample size, were based on previous studies investigating the postprandial decline in ghrelin  
367 level <sup>5,32,35-38</sup>. However, such experimental design may have prevented us from detecting the  
368 postprandial change of ghrelin proteolytic activity in individuals with OW/OB, if an increase  
369 in such enzymatic activity takes place with a different kinetics or if it requires a higher sample  
370 size to be unmasked. Also, the time chosen to collect the postprandial blood sample did not  
371 allow us to detect the transient meal-induced increase of glycemia and, consequently, we  
372 could not evaluate if postprandial hyperglycaemia either was differentially affected in  
373 individuals with OW/OB or correlated the postprandial increase of ghrelin proteolytic activity  
374 in lean individuals. The study did not include “sham-feeding” control groups and,  
375 consequently, a postprandial increase of ghrelin proteolytic activity may be related to its

376 physiological diurnal rhythm. Despite these limitations, the current study was very valuable  
377 to gain insights about a novel aspect of the biology of ghrelin in human plasma.

378

379 Ghrelin des-acylation in plasma is fast and affects the circulating ghrelin level <sup>50</sup>.  
380 Indeed, intravenous administration of ghrelin in humans results in a peak of ghrelin levels,  
381 at 15 min after injection, and is rapidly eliminated <sup>11,40</sup>. Ghrelin des-acylation seems to be  
382 the main mechanism mediating the reduction of plasma ghrelin elevation since the referred  
383 increase of ghrelin level is followed by a peak of DAG level, which then disappears from  
384 plasma more slowly than ghrelin <sup>11,40</sup>. Here, we found that ghrelin des-acylation was  
385 unaffected in individuals with OW/OB or postprandially suggesting that des-acylation is not  
386 a key mechanism mediating the decrease of plasma ghrelin level in those metabolic  
387 conditions. In line with our observations, it was reported that the level of serum  
388 butyrylcholinesterase activity, a ghrelin esterase in humans, was not altered during fasting  
389 <sup>51</sup>. Since our results indicate that ghrelin proteolysis is an important mechanism affecting the  
390 decrease of plasma ghrelin level in males with OW/OB, the mechanisms mediating the  
391 decrease of plasma ghrelin level in females with OW/OB remain uncertain. Given the fast  
392 rate of ghrelin des-acylation, it can be hypothesized that the observed decrease of plasma  
393 ghrelin level in the above referred conditions is due to a reduction of the secretion of ghrelin  
394 from the stomach <sup>10</sup>, as it has been shown in obese mice <sup>52</sup>. Also, it is possible that des-  
395 acylation of circulating ghrelin is unaffected in plasma but regulated in some specific tissues,  
396 such as liver. Future studies in humans are needed to test these possibilities.

397

## 398 **Conclusions**

399 We provide here evidence indicating that ghrelin proteolysis in plasma increases in  
400 males with OW/OB and that it is acutely regulated by food intake in lean individuals. Also,  
401 we report that ghrelin des-acylation in plasma is not affected by body weight or food intake.  
402 Thus, current study helps to better understand the mechanisms controlling the changes of  
403 plasma ghrelin under different metabolic conditions in humans. Ghrelin proteolysis may be  
404 a putative pharmacological target to control circulating ghrelin level in humans. In particular,  
405 enhancement of ghrelin proteolysis in males with OW/OB or after food intake may help to  
406 further reduce plasma ghrelin level, with concomitant “beneficial” effects such as appetite  
407 reduction and downregulation of mechanisms that increase glycemia.

408

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418

419 **Conflict of interest**

420 The authors declare no conflicts of interest. The sponsors had no role in the design of the  
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581

582 **Figure Legends.**

583

584 Figure 1. Overview of methods to assess ghrelin des-acylation (A) and ghrelin proteolysis  
585 (B). See text for details. Eppendorf tube illustration adapted from scidraw.io  
586 (doi.org/10.5281/zenodo.6808872).

587

588 Figure 2. Preprandial plasma levels of ghrelin (A), DAG (B), ghrelin/DAG ratio (C), ghrelin  
589 des-acylation activity (D) and ghrelin proteolysis (E) in NW and OW/OB male participants.  
590 \*, \*\*, \*\*\* and \*\*\*\* indicate significant difference at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.0001$  by  
591 Student's t test or Mann Whitney test.

592

593 Figure 3. Correlations of ghrelin proteolysis with ghrelin (A), DAG (B), BMI (C), and glycemia  
594 (D) in male participants in preprandial conditions.

595

596 Figure 4. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B), ghrelin des-  
597 acylation activity (C) and ghrelin proteolysis (D) in males with NW or OW/OB. Coloured  
598 points and lines show the geometric mean and each grey line represents the change within  
599 an individual participant. Exact P values are shown. Two-way repeated measures analysis  
600 of variance (ANOVA) with Bonferroni multiple-comparisons test was performed. \*: significant  
601 difference vs. same group but different feeding condition. #: significant difference vs.  
602 different group but same feeding condition.

603

604 Figure 5. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B) and ghrelin  
605 proteolysis (C) in females with NW or OW/OB. Coloured points and lines show the geometric  
606 mean and each grey line represents the change within an individual participant. Exact P  
607 values are shown. Two-way repeated measures analysis of variance (ANOVA) with  
608 Bonferroni multiple-comparisons test was performed. \*: significant difference vs. same group  
609 but different feeding condition. #: significant difference vs. different group but same feeding  
610 condition.

**Table 1: Baseline features of male participants**

	<b>Participants with NW</b>	<b>Participants with OW/OB</b>	<b>p-value</b>
Age	34.7 ± 9.1	37.1 ± 5.0	N.S.
BMI	23.6 ± 0.9	30.9 ± 4.6	0.0001
Glycemia (g L <sup>-1</sup> )	0.99 ± 0.04	1.08 ± 0.07	0.0042
Insulin (μIU mL <sup>-1</sup> )	6.29 ± 3.21	14.28 ± 8.35	0.0139
HOMA index	1.55 ± 0.81	3.83 ± 2.31	0.0114

Abbreviations: BMI: body mass index; HOMA: homeostatic model assessment.

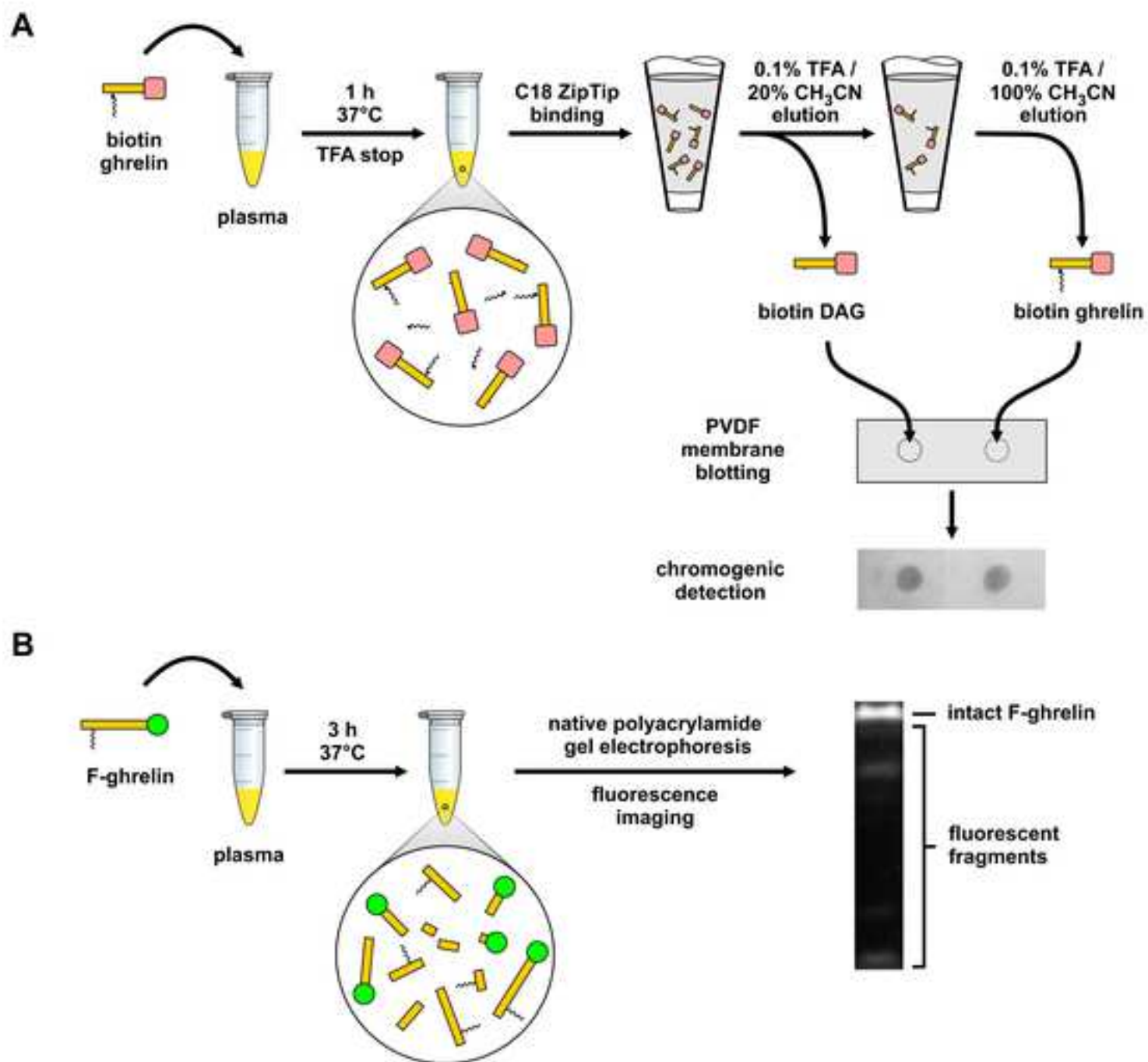
Data are presented as mean ± SD. Data analysis performed by t-test.

**Table 2: Baseline features of female participants**

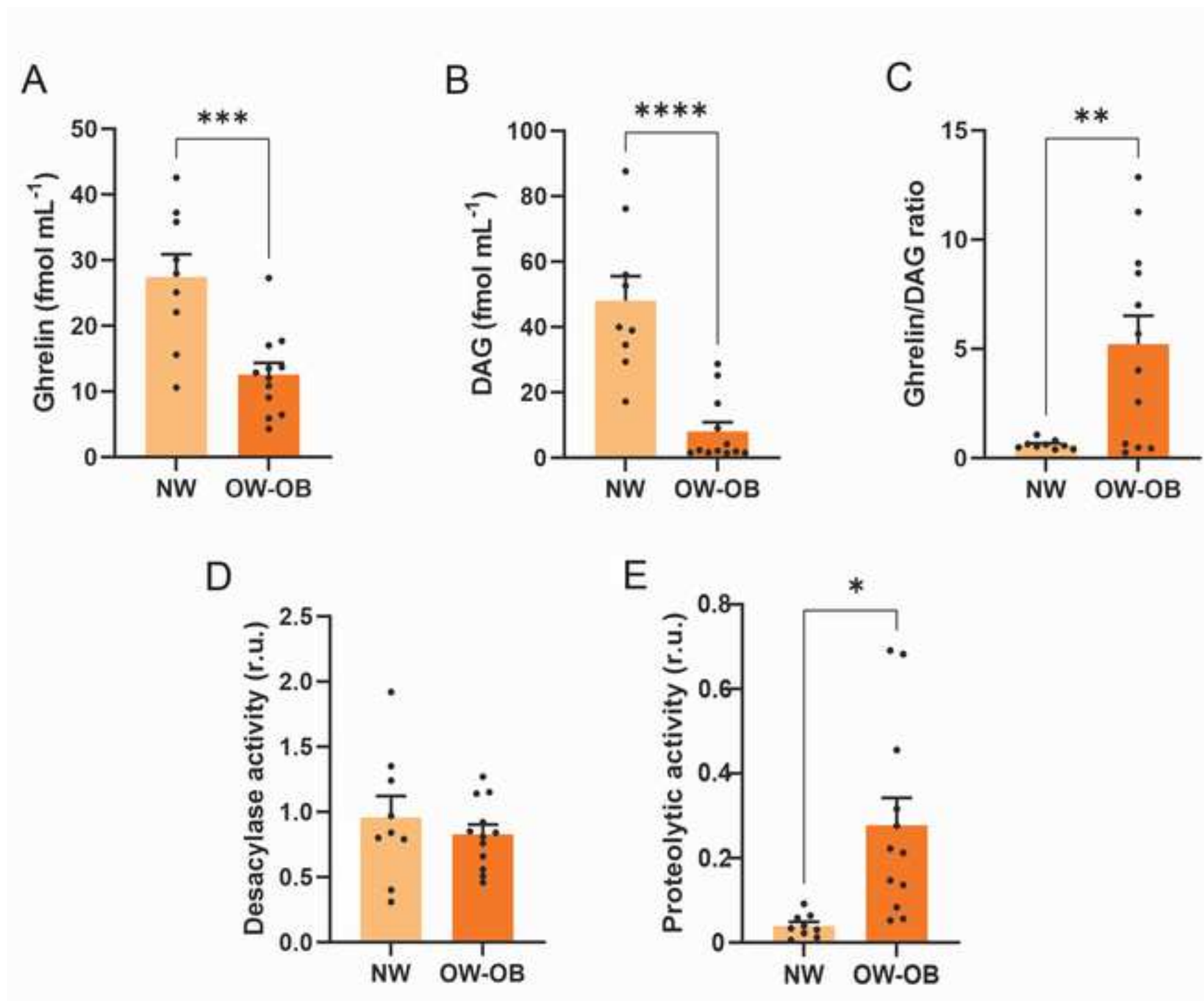
	Participants with NW	Participants with OW/OB	p-value
Age	34.5 ± 8.1	30.3 ± 7.9	N.S.
BMI	22.3 ± 2.1	29.1 ± 2.4	<0.0001
Glycemia (g L <sup>-1</sup> )	0.96 ± 0.05	0.98 ± 0.04	N.S.
Insulin (μIU mL <sup>-1</sup> )	5.15 ± 1.41	12.87 ± 4.42	<0.0001
HOMA index	1.22 ± 0.35	3.13 ± 1.11	<0.0001
Ghrelin (fmol mL <sup>-1</sup> )	40.26 ± 20.56	19.47 ± 9.60	0.0180
DAG (fmol mL <sup>-1</sup> )	63.77 ± 27.16	24.45 ± 9.27	0.0011
Ghrelin/DAG ratio	0.61 ± 0.21	0.77 ± 0.22	N.S.
Proteolytic activity (r.u.)	0.04 (0.02; 0.10)	0.06 (0.03; 0.21)	N.S.
Desacylase activity (r.u.)	1.31 ± 0.77	0.94 ± 0.12	N.S.

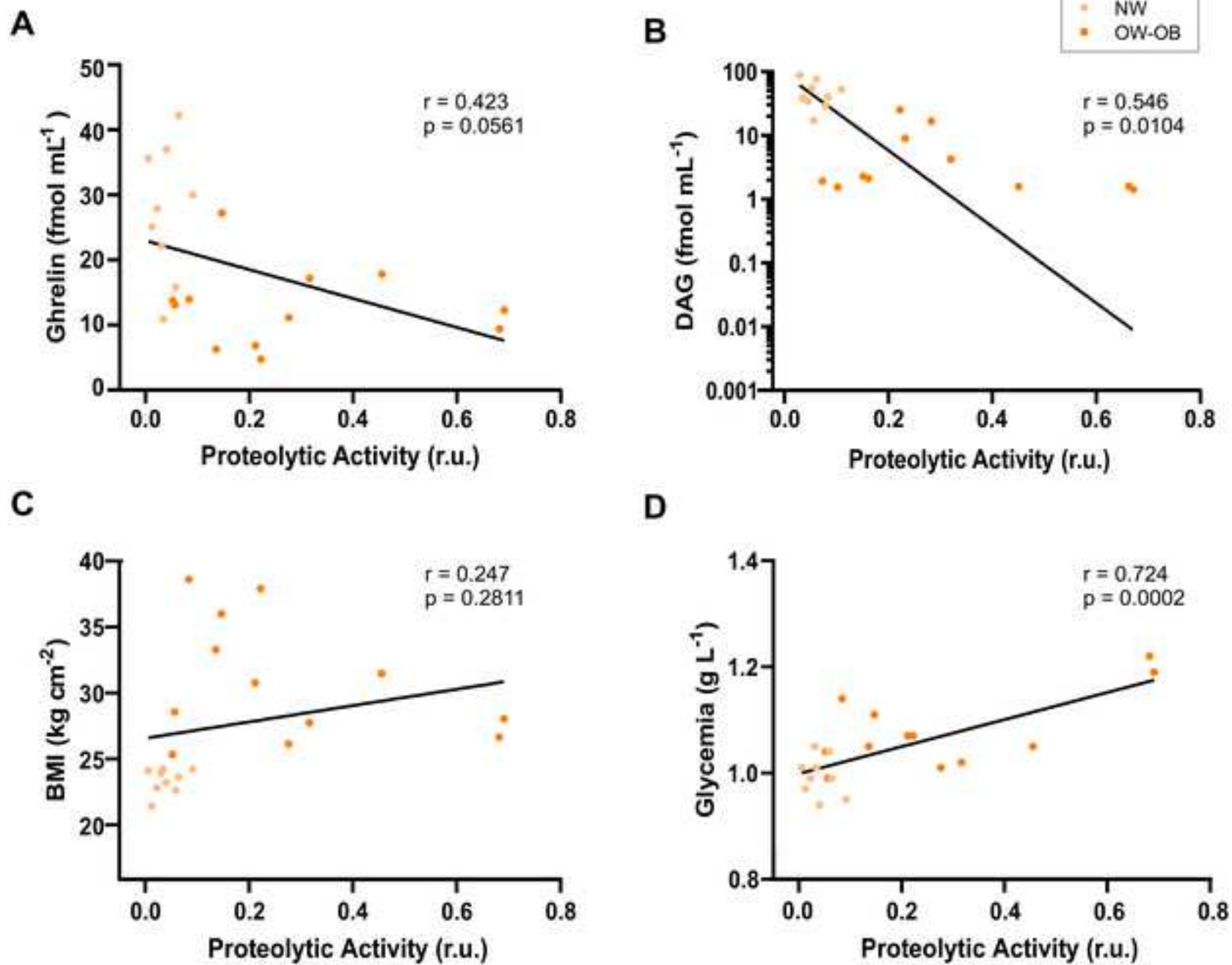
Abbreviations: BMI: body mass index; HOMA: homeostatic model assessment; DAG: desacyl-ghrelin.

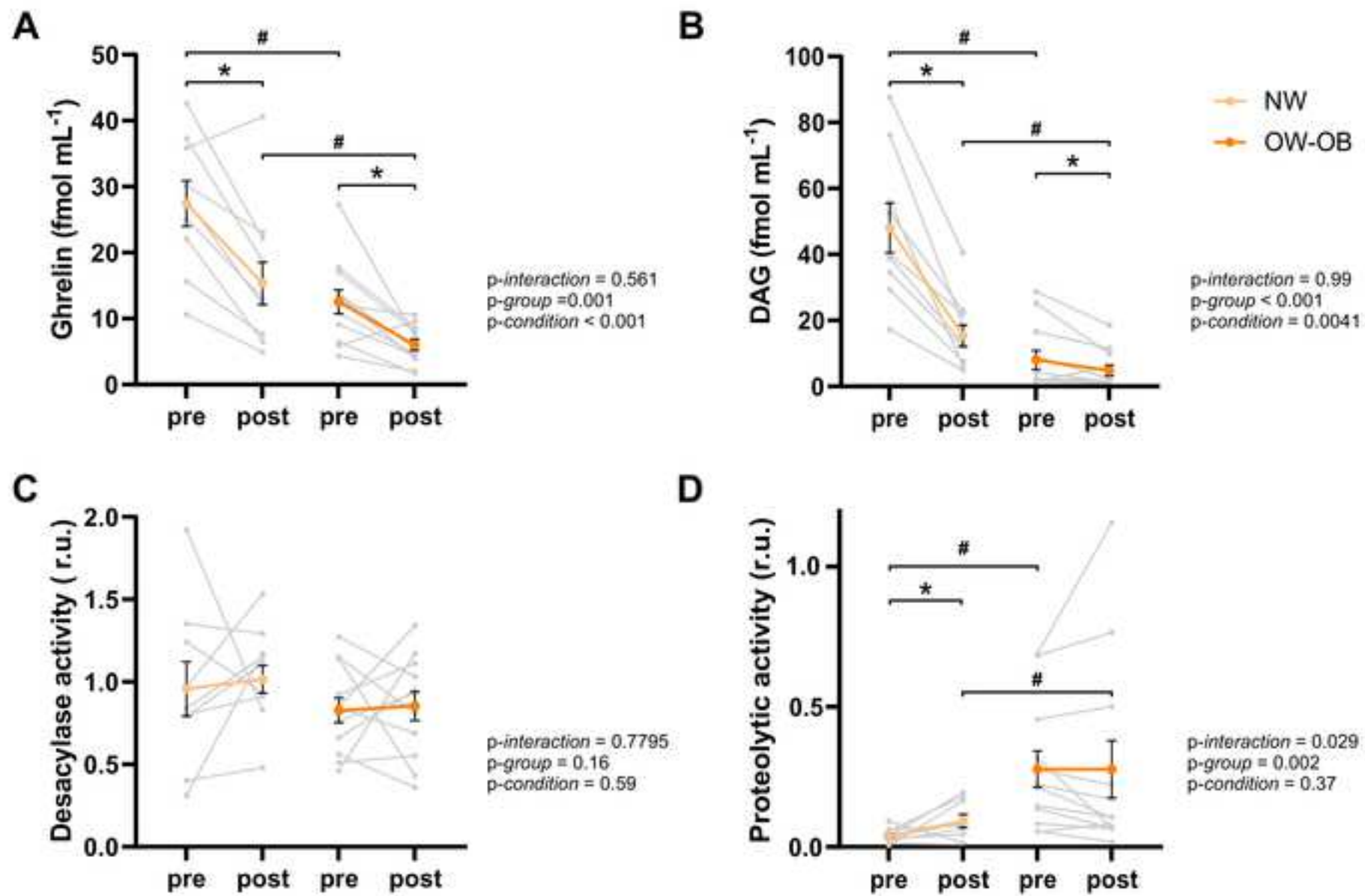
Data are presented as mean ± SD and analysed by t-test for all parameters except for proteolytic activity, which is presented as median (IQR) and analysed by Mann Whitney test.

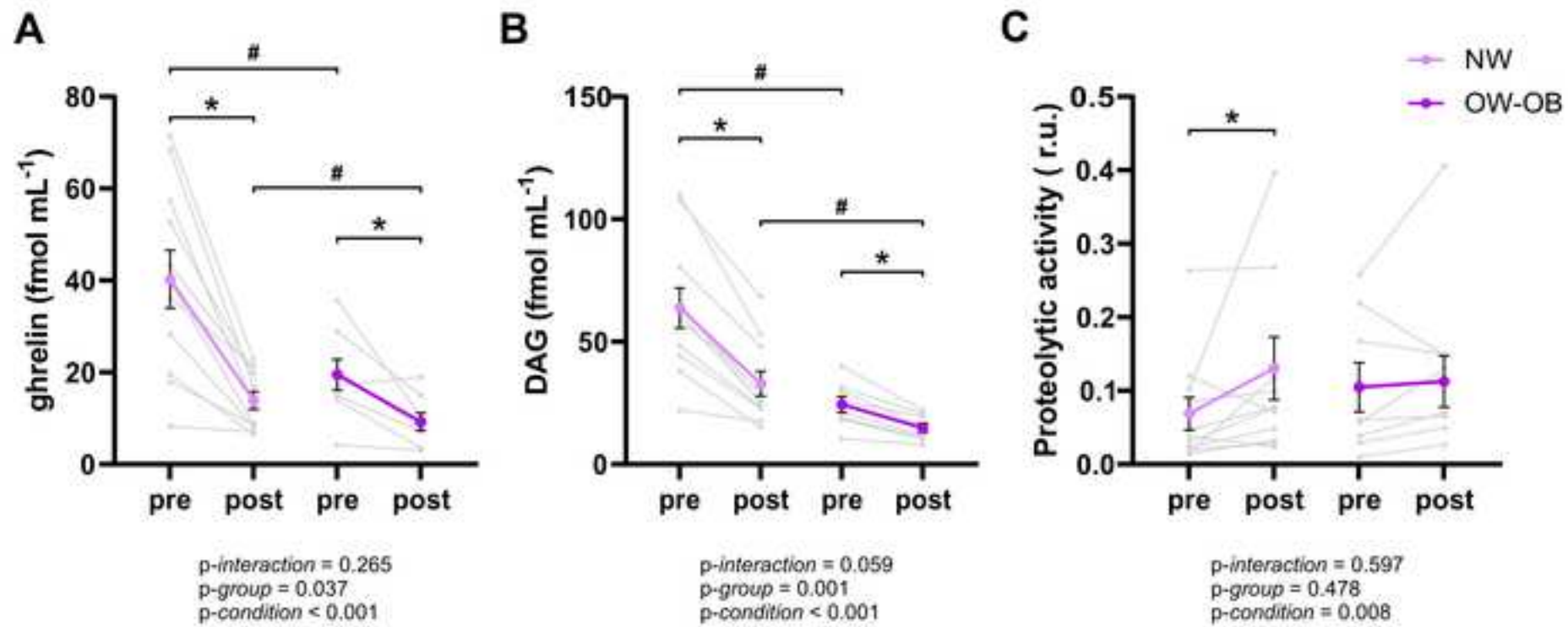












**Credit author statement**

Conceptualization, formal analysis, original draft preparation, supervision, overall responsibility for the project: MFA, MP.

Methodology, validation, formal analysis, investigation, resources and data curation; ASF, DC, DL, CS, PPDF, CRT, TL, LGL, SC, JAF, MFA, MP.

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