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1 **Growth and Differentiation Factor 15 is secreted by skeletal muscle during exercise and**
2 **promotes lipolysis in humans**

3

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28 **Abstract**

29 We hypothesized that skeletal muscle contraction produces a cellular stress signal triggering
30 adipose tissue lipolysis to sustain fuel availability during exercise. The present study aimed at
31 identifying novel exercise-regulated myokines, also known as exerkinines, able to promote
32 lipolysis.

33 Human primary myotubes from lean healthy volunteers were submitted to electrical pulse
34 stimulation (EPS) to mimic either acute intense or chronic moderate exercise. Conditioned
35 media (CM) experiments with human adipocytes were performed. Conditioned media and
36 human plasma samples were analyzed using unbiased proteomic and/or ELISA. Real-time
37 qPCR was performed in cultured myotubes and muscle biopsy samples.

38 CM from both acute intense and chronic moderate exercise increased basal lipolysis in human
39 adipocytes (1.3 to 8 fold, $p < 0.001$). Growth and Differentiation Factor 15 (GDF15) gene
40 expression and secretion increased rapidly upon skeletal muscle contraction. GDF15 protein
41 was up-regulated in CM from both acute and chronic exercise-stimulated myotubes. We
42 further show that physiological concentrations of recombinant GDF15 protein increase
43 lipolysis in human adipose tissue, while blocking GDF15 with a neutralizing antibody
44 abrogates EPS CM-mediated lipolysis.

45 We herein provide the first evidence that GDF15 is a novel exerkinine produced by skeletal
46 muscle contraction and able to target human adipose tissue to promote lipolysis.

47

48 **Keywords:** Myokine; Exercise; Adipose Tissue; Lipolysis; Skeletal Muscle Cells

49 **Introduction**

50 Exercise improves metabolic health and prevents the complications of obesity and
51 type 2 diabetes (1, 2). This is partly due to the release of secreted factors by skeletal muscle,
52 i.e. myokines, which can virtually target all organs remotely (3). Over the last decade,
53 hundreds of proteins secreted by skeletal muscle have been identified as reviewed in details
54 elsewhere (4-7). However, very few myokines have been shown to target white adipose tissue
55 particularly in humans. In a previous clinical study, we observed a remarkably enhanced *in*
56 *situ* and systemic lipolytic response in lean healthy endurance-trained subjects performing a
57 high intensity exercise the day after an exhaustive glycogen-depleting exercise compared to
58 rest (8). This greater lipolytic response could not be explained by significant changes in
59 classically known lipolytic stimuli such as increased catecholamines, atrial natriuretic peptide,
60 growth hormone, cortisol, interleukin 6 or a decrease in circulating insulin during exercise.
61 We therefore hypothesized that skeletal muscle contraction in the context of acute high
62 intensity exercise and chronic moderate exercise may produce a cellular stress signal capable
63 to increase adipose tissue lipolysis to sustain fuel availability and delay muscle glycogen
64 depletion.

65 Recently, Growth and Differentiation Factor 15 (GDF15) emerged as a biomarker of
66 cellular stress that can be produced by a number of organs such as lung, kidney and liver (9-
67 11). GDF15, also known as macrophage inhibitory cytokine-1 (MIC-1), is a stress-induced
68 cytokine and an ancient member of the transforming growth factor beta superfamily. High
69 levels of circulating GDF15 have been found in various diseases states such as cancer, heart
70 failure and mitochondrial diseases (12, 13). Very recent studies demonstrate a major role of
71 GDF15 in appetite suppression through non canonical brain neuronal networks in the
72 brainstem such as the area postrema and the nucleus tractus solitaries (14-17). Injection of
73 recombinant GDF15 protein in mice fed a high fat diet (HFD) induces robust weight loss (16).

74 Similarly GDF15-overexpressing mice are resistant to HFD-induced obesity and metabolic
75 disturbances (18). These effects appear largely mediated by food intake suppression through
76 the GDF15 cognate receptor GFRAL, GDNF receptor alpha-like, which heterodimerizes with
77 a co-receptor called RET (15, 17). RET encodes a tyrosine kinase receptor for members of the
78 glial cell line-derived neurotrophic factor (GDNF) family (15). In mice, GFRAL expression
79 appears strictly confined to the area postrema and the nucleus of the tractus solitarius. It is
80 suggested that GDF15 behaves as a metabolic stress signal in response to nutritional stress to
81 trigger conditioned taste aversion (11).

82 We herein describe GDF15 as a novel exercine of a peripheral crosstalk between
83 skeletal muscle and adipose tissue. We show that conditioned media from exercised human
84 myotubes activate lipolysis in human adipocytes *in vitro*. Through proteomic screening of the
85 conditioned media, we next identified GDF15 as a novel exercise-regulated myokine that is
86 rapidly released upon muscle contraction. Neutralization of GDF15 in conditioned media
87 abolishes lipolysis while treatment of adipose tissue explants with recombinant GDF15
88 promotes lipolysis. Collectively, we herein provide the first evidence that GDF15 is produced
89 by skeletal muscle upon contraction and that it is able to promote lipolysis in human adipose
90 tissue.

91 **Results**

92 **Conditioned media from exercised myotubes trigger lipolysis in adipocytes**

93 The physiological and metabolic adaptations of skeletal muscle to exercise are well known
94 (1). Using electrical pulse stimulation (EPS) of human myotubes to induce forced contraction,
95 we modeled two exercise paradigms: an acute high intensity exercise model (EPS3h) and a
96 chronic moderate exercise training model (EPS24h). EPS activates canonical exercise
97 signaling pathways such as p38 mitogen-activated protein kinases (p38 MAPK) and
98 Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) up to 48h (Figure S1A). As expected,
99 EPS3h which mimics an acute high intensity exercise induced a pronounced glycogen
100 depletion (Figure 1A) and concomitant increase in lactate production (~4 fold, p<0.01)
101 (Figure 1B). Consistent with this type of exercise, no major change in glucose oxidation rate
102 was observed (Figure 1C), while lipid oxidation rate was reduced by 35% (Figure 1D). EPS3h
103 also induced gene expression of classically known myokines such as interleukin 6 (IL6), IL15
104 and fibroblast growth factor 21 (Figure S1B).

105 We further validated that EPS24h, which mimics chronic moderate exercise training, did not
106 significantly reduced glycogen content (Figure 1E) while increasing basal glycogen storage
107 capacity (~1.5 fold, p<0.05) (Figure 1F), glucose oxidation rate (~2 fold, p<0.05) (Figure 1G),
108 and lipid oxidation rate (~3 fold, p<0.05) (Figure 1H). EPS24h also increased lactate
109 production (2.2-fold, p<0.01) in a lower range than EPS3h (Figure S2A). EPS24h increased
110 IL6 mRNA level (~3 fold, p<0.01), slightly induced myostatin, and decreased Fibronectin
111 Type III Domain Containing 5 (FNDC5) and Brain Derived Neurotrophic Factor (BDNF)
112 mRNA levels (Figure S1C). To test our hypothesis that skeletal muscle contraction produces a
113 cellular stress signal that trigger lipolysis to sustain fuel availability during exercise, we next
114 incubated human multipotent adipose-derived stem (hMADS) adipocytes with conditioned

115 media (CM) of EPS-stimulated myotubes (Figure 1I). Interestingly, CM from EPS3h-
116 stimulated myotubes induced a modest increase of basal lipolysis reflected by glycerol
117 production (~1.3 fold, $p < 0.001$) in the absence of any lipolytic stimuli (Figure 1J). In the same
118 line, CM from EPS24h-stimulated myotubes induced a more robust basal lipolysis (~3 fold,
119 $p < 0.001$) (Figure 1K). Similarly, CM from EPS24h induced a significant increase of NEFA
120 release (1.7 fold, $p < 0.05$) (Figure S2B).

121 In summary, exercise-mediated skeletal muscle contraction produces secreted factors able to
122 activate lipolysis in adipocytes *in vitro*.

123

124 **GDF15 is a novel exerkin rapidly induced by skeletal muscle contraction**

125 To identify potential secreted proteins produced by skeletal muscle contraction, we performed
126 a proteomic screen of CM from EPS-stimulated and non-stimulated myotubes (Figure 2A &
127 D). We quantified 1311 classically and non classically secreted proteins. Among the
128 significantly up-regulated proteins, GDF15 caught our attention (Figure 2A), as recent studies
129 described GDF15 as a novel metabolic hormone (14-17). GDF15 protein levels were
130 increased by about 2.5 fold in EPS3h CM (Figure 2B). We confirmed this finding by ELISA,
131 GDF15 increased from 21.7 ± 2.1 pg/ml to 29.6 ± 3.4 pg/ml in EPS3h CM ($p < 0.01$) (Figure 2C).
132 Importantly, we also identified GDF15 as a significantly up-regulated protein in EPS24h CM
133 (3 fold, $p < 0.05$) (Figure 2D-F). The concentration of GDF15 in EPS24h CM ranged from 19.4
134 to 110.9 pg/ml and was nearly 2-fold higher (41.2 ± 4.0 vs 21.7 ± 2.1 pg/ml) compared to EPS3h
135 CM. Time-course of EPS in human primary myotubes showed that GDF15 is significantly up-
136 regulated within 1h (Figure 2G). Overall, we here identify GDF15 as a novel exercise-
137 regulated myokine.

138

139 **GDF15 gene expression is responsive to skeletal muscle contraction and exercise**
140 **mimetics**

141 Since GDF15 is a stress-responsive hormone, we tested whether acute EPS can modulate
142 GDF15 mRNA levels. We observed a rapid up-regulation of GDF15 mRNA levels within
143 30min exposure to EPS (Figure 3A). A second peak of mRNA levels was observed at 3h EPS.
144 In agreement with protein secretion data, we observed a significant up-regulation of GDF15
145 mRNA levels for EPS3h-stimulated myotubes (1.7 fold, $p<0.01$) (Figure 3B). We similarly
146 observed an up-regulation of GDF15 mRNA levels in EPS24h-stimulated myotubes (Figure
147 3C). Since exercise activates a complex integrative response including neuroendocrine
148 changes beyond muscle contraction, we assessed the influence of exercise-mimetics on
149 GDF15 mRNA levels. Forskolin which increases intracellular levels of the second messenger
150 cAMP, did not change GDF15 gene expression (Figure 3D), while interestingly both
151 ionomycin which facilitates calcium release (Figure 3E) and the peroxisome proliferator-
152 activated receptor β agonist GW0742 (Figure 3F) increased GDF15 mRNA levels (1.5-2 fold,
153 $p<0.01$). We next investigated GDF15 mRNA levels in muscle biopsy samples from lean
154 healthy individuals in response to 1h of acute exercise at 55% peak power output on a bicycle
155 ergometer (Human study 1). We found that GDF15 mRNA levels increase up to 18-fold in
156 skeletal muscle in response to exercise (Figure 3G).

157

158 **GDF15 plasma levels in response to exercise, obesity and lifestyle**

159 Since our *in vitro* work revealed GDF15 as a novel exercine, we measured GDF15 in plasma
160 of lean healthy individuals randomly assigned in a crossover design to either 1h of endurance

161 exercise performed at 60% of VO₂peak or sprint interval exercise consisting of 7 repetitions
162 of 30s at 130% of maximal aerobic power (Human study 2) (Figure 4A-C). In line with a
163 recent report (19), we observed that both type of exercise, endurance (Figure 4A) and sprint
164 interval (Figure 4B), augment plasma GDF15 circulating levels. Interestingly, the exercise
165 response (exercise minus rest) tended to be lower ($p=0.07$) for the sprint interval exercise
166 which produces overall a lower energy expenditure and energetic stress despite a greater
167 power output during very short periods of time (30s) (Figure 4C).

168 We further show that acute endurance exercise (45 min at 50% VO₂max) raises plasma
169 GDF15 levels in both middle-aged and elderly obese subjects (Human study 3) (Figure 4D &
170 E). Plasma GDF15 levels were measured at rest and immediately after exercise at baseline
171 and in response to an 8-week lifestyle intervention program combining moderate calorie
172 restriction and aerobic and resistance exercise. Obese subjects lost in average 5.1 ± 0.5 kg post-
173 lifestyle intervention (data not shown). Exercise-induced increase of plasma GDF15 was
174 observed in both groups at baseline and post-lifestyle (Figure 4D & E). When combining all
175 subjects, we observed a slight but significant increase of plasma GDF15 levels in response to
176 lifestyle-induced weight loss (Figure 4F). This increase of plasma GDF15 tended to be
177 positively correlated with lifestyle-induced weight loss in elderly obese subjects ($r=0.66$;
178 $p=0.07$). Lean healthy and middle-aged obese subjects had plasma GDF15 levels within the
179 same range (Figure 4G), while BMI-matched elderly obese had higher circulating levels of
180 GDF15 compared to middle-aged obese individuals (Figure 4H). Consistent with this finding,
181 we observed a significant positive correlation between resting plasma GDF15 levels and age
182 in Human study 3 ($r=0.512$, $p=0.043$). Interestingly, no sex-difference in resting plasma
183 GDF15 levels were found in Human study 4 (men: 475 ± 56 vs women: 472 ± 46 pg/ml).

184 Collectively, we here show that plasma GDF15 responds to various modalities of exercise in
185 lean and obese individuals, and increases with aging.

186

187 **GDF15 targets human adipose tissue to promote lipolysis**

188 We noted a positive correlation between plasma glycerol and GDF15 level in all obese
189 subjects investigated at rest and during exercise (Human study 3) (Figure S3A). We further
190 observed a nearly significant positive relationship ($r=0.35$, $p=0.067$) between changes in
191 plasma GDF15 levels and changes in BMI in obese individuals in response to very low calorie
192 diet-induced weight loss (Human study 4) (Figure S3B). We therefore hypothesized that
193 GDF15 was able to target white adipose tissue to induce lipolysis and weight loss. We first
194 examined GDF15 receptors GFRAL and RET mRNA levels in human abdominal adipose
195 tissue biopsy samples (Human study 4). We found a significant expression of the GDF15
196 cognate receptor GFRAL in whole adipose tissue fraction as well as in isolated adipocytes
197 (Figure S4A). Interestingly, GFRAL expression was virtually null in whole human muscle
198 tissue. The co-receptor RET followed a similar pattern of expression as GFRAL in adipose
199 tissue except for whole skeletal muscle tissue where its expression was comparable to whole
200 adipose tissue (Figure S4B). As both GFRAL and RET were expressed to comparable levels
201 in adipocytes and adipose stroma vascular cells, we further examined both GDF15 receptor
202 expression in sorted stromal cells. We observed that both GFRAL (Figure S4C) and RET
203 (Figure S4D) were predominantly expressed in preadipocytes, again confirming a metabolic
204 role of GDF15 in adipocytes. Neither GFRAL (Figure S4E) nor RET (Figure S4F) adipose
205 gene expression were influenced by obesity grade. However they were both associated with a
206 number of biological and clinical variables such as plasma triglycerides, HOMA-IR and
207 Matsuda index, while adipose RET mRNA levels were inversely related to BMI (Table 1).

208 We then investigated the effect of recombinant human GDF15 (rhGDF15) protein on lipolysis
209 in human abdominal adipose tissue explants. rhGDF15 significantly modestly increased

210 lipolysis at the physiological concentration of 1 ng/ml (~10%, $p < 0.05$), reflected by both
211 glycerol (Figure 5A) and NEFA release (Figure 5B). This lipolytic response was weak
212 compared to those observed for forskolin and the non selective β -adrenergic agonist
213 isoprenaline which increased lipolysis to about 2-3 fold. A higher supra-physiological
214 concentration of rhGDF15 at 100 ng/ml did not further raised basal lipolysis (~11%, $p < 0.05$)
215 in adipose explants from 6 independent donors (Figure 5C-D). Even more remarkable, we
216 demonstrate that blocking GDF15 with a neutralizing IgG antibody of hGDF15 completely
217 abrogates the lipolytic response of EPS24h CM (Figure 5E), thus indicating that GDF15
218 likely triggers most, if not all, the EPS24h CM lipolytic effect in hMADS adipocytes. To
219 validate this finding, we verified that incubating a non-specific IgG antibody in control wells
220 did not affect basal lipolysis (data not shown). We finally wondered whether this lipolytic
221 effect of GDF15 was a human specificity. We measured both GFRAL and GDF15 gene
222 expression in whole brain, perigonadic white adipose tissue and skeletal muscle of adult
223 female mice. Although GDF15 was expressed in all three organs (Figure S5A), GFRAL was
224 only detectable in brain (Figure S5B), thus confirming recent findings (16).

225 Altogether, our data provide the first evidence that GDF15 can target human adipose tissue to
226 trigger lipolysis.

227 **Discussion**

228 Investigators have been searching for a link between muscle contraction and metabolic
229 changes in other organs such as the liver and the adipose tissue for a few decades. The targets
230 of an ‘exercise factor’ have been humoral proteins released from skeletal muscle during
231 contraction that mediate the adaptations of chronic exercise training (4, 20). This has opened a
232 novel paradigm that skeletal muscle is an endocrine organ producing and releasing hormones,
233 i.e. myokines, which exerts endocrine effects on remote organs (4, 21). We here provide
234 compelling evidence that GDF15 behaves as a novel exerkine involved in a crosstalk between
235 skeletal muscle and adipose tissue (Figure 5F). We show that the cellular stress mediated by
236 skeletal muscle contraction can be translated into a systemic response through GDF15-
237 mediated lipolysis in white adipocytes.

238 As we hypothesized that skeletal muscle contraction can release a cellular stress signal
239 able to induce lipolysis, we developed an experimental model of exercise in cultured human
240 myotubes. Human primary culture of skeletal muscle cells has been widely used by several
241 investigators (22-26) and provides a powerful tool for mechanistic studies of skeletal muscle
242 and to study muscle autonomous adaptations in the absence of the systemic milieu (27). EPS
243 of human myotubes can be used to induce forced contraction and mimic exercise *in vitro* (28).
244 Although some studies have used EPS to assess individual variability in biological response
245 to exercise *in vitro* (29, 30) and to identify novel secreted factors by skeletal muscle
246 contraction (31-33), very few have investigated how closely EPS recapitulates the metabolic
247 consequences of exercise. We here developed two exercise models: one acute high intensity
248 exercise that recapitulates the expected physiological responses with strong glycogen
249 depletion and high lactate production (34), and one chronic moderate exercise which more
250 closely mimics the biological adaptations of endurance exercise training with increased basal
251 glycogen storage along with an up-regulation of substrate oxidation rates (35).

252 To identify potential muscle contraction-derived lipolytic factors, we analyzed CM
253 from our two exercise models with human adipocytes. Remarkably, CM from both exercise
254 models increased basal lipolysis. This effect was quite modest for the acute intense exercise
255 model but more pronounced for the chronic moderate exercise model. Thus a muscle
256 contraction-derived factor contained in CM has the potential to promote lipolysis. Among the
257 currently known myokines, only IL6 has been previously suggested to induce lipolysis in
258 humans (36). Later the same authors proposed that muscle-derived IL6 does not promote fat
259 mobilization from subcutaneous adipose tissue, at least at moderate exercise intensity (37),
260 but rather selectively stimulates fat metabolism in human skeletal muscle (38). This
261 observation was corroborated by *in vitro* work in adipose tissue explants showing that IL6
262 inhibits lipoprotein lipase activity (39). Altogether most evidence so far indicates that IL6
263 does not acutely stimulate lipolysis in human adipose tissue. In order to unravel novel putative
264 lipolytic myokines, we performed a proteomic screen of EPS CM and identified GDF15 as a
265 significantly up-regulated protein in our two *in vitro* exercise models. GDF15 mRNA levels
266 rise within 30min in response to muscle contraction, followed by an elevated secretion by 1h.
267 GDF15 gene expression was also induced by pharmacological exercise-mimetics such as
268 ionomycin and the PPAR β agonist GW0742, but remained insensitive to activation of cAMP-
269 signaling by forskolin. This indicates that other molecular pathways related to exercise are
270 involved in GDF15 regulation beyond muscle contraction. As GDF15 is a stress-responsive
271 factor, components of the integrated stress response pathway including the protein kinase R
272 (PKR)-like endoplasmic reticulum kinase (PERK), the eukaryotic translation initiation factor
273 2-alpha (EIF2 α), the activating transcription factor 4 (ATF4) and the CCAAT-enhancer-
274 binding protein homologous protein (CHOP) may be involved (11). A few studies have
275 already suggested that this integrated stress response pathway is activated by exercise (40,

276 41). Thus several molecular pathways may be involved in the regulation of GDF15
277 expression/secretion in skeletal muscle.

278 We consolidated our *in vitro* findings showing that GDF15 mRNA levels rise from 2-
279 to 20-fold in skeletal muscle of lean healthy subjects in response to acute aerobic exercise.
280 This local rise of GDF15 in skeletal muscle was paralleled by an increase of circulating
281 GDF15 levels during endurance and sprint interval exercise in lean healthy volunteers.
282 Although circulating GDF15 can originate from other tissue sources, it is tempting to
283 speculate, based on our data, that contracting skeletal muscle contributes to increase
284 circulating GDF15 levels during exercise. This is in contrast with a recent study suggesting
285 that the rise of circulating GDF15 during exhaustive exercise appears independent of skeletal
286 muscle (19). Alternatively, muscle-derived GDF15 could play a paracrine role within skeletal
287 muscle to target intramuscular adipose tissue. Obesity did not alter exercise-induced
288 circulating GDF15, but interestingly, lifestyle-induced weight loss in middle-aged and elderly
289 obese individuals significantly increased baseline circulating GDF15 levels. The rise of
290 plasma GDF15 levels was positively related to weight loss in response to the lifestyle
291 program in elderly obese people, again highlighting a link between GDF15 and adipose tissue
292 loss. Emmerson et al. observed that human subcutaneous adipose tissue is a significant site of
293 expression of the GDF15 receptor GFRAL (16). We therefore went on to hypothesize that
294 GDF15 could target adipose tissue to promote lipolysis.

295 GDF15 is a cellular stress-responsive hormone that has recently gained much interest
296 as anti-obesity therapy (42, 43). Recent studies indicate that GDF15 binds to a heterodimeric
297 receptor complex composed of GFRAL and RET which expression is restricted to the
298 brainstem in mice (14-17). We here provide evidence that GFRAL and RET are significantly
299 expressed in human adipocyte. A comparable expression is also found in the stroma vascular
300 fraction of adipose tissue, and further cell-type specific analyses revealed that both GFRAL

301 and RET are predominantly expressed in preadipocytes compared to macrophages and
302 lymphocytes. In contrast, GFRAL expression in skeletal muscle tissue is virtually null thus
303 excluding a potential autocrine role of GDF15. Our data are consistent with the Genotype-
304 Tissue Expression (GTEx) public database indicating that GFRAL is predominantly
305 expressed in human subcutaneous adipose tissue. Of interest, GFRAL and RET gene
306 expression in subcutaneous abdominal adipose tissue is not influenced by adiposity and
307 obesity grade. Adipose GFRAL and RET gene expression correlated positively with plasma
308 triglycerides, HOMA-IR and Matsuda index. This is consistent with the recent observation
309 that GDF15 behaves as a nutritional stress signal induced by long-term high fat feeding and
310 metabolic disturbances in mice (9-11). In line with an effect of GDF15 on adipose tissue,
311 adipose RET gene expression was inversely related to BMI. Consistently, we here show that a
312 physiological concentration of recombinant human GDF15 induces lipolysis in a comparable
313 range as forskolin in human subcutaneous abdominal adipose tissue explants. In our clinical
314 studies and other studies, circulating GDF15 typically ranged between 0.3 and 1 ng/ml (43,
315 44). Higher supra-physiological concentrations up to 100 ng/ml, as observed in cancer
316 cachexia (44), did not further increase lipolysis. Most importantly, neutralization of GDF15 in
317 EPS24h CM with a monoclonal antibody completely abrogated lipolysis, implying that
318 GDF15 triggers most if not all the lipolytic response in CM from EPS-stimulated human
319 myotubes. The higher lipolytic response observed in EPS24h CM could be explained by the
320 nearly 2-fold greater concentration of GDF15 accumulated in the medium over 24h.
321 Adipocyte lipolysis is a major physiological process of fuel supply in conditions of increased
322 energy needs such as exercise (45, 46). Our work identifies GDF15 as a novel homeostatic
323 hormone of the crosstalk between contracting skeletal muscles and adipose tissue in humans.

324 In summary, GDF15 is a novel 'exercise factor' involved in a homeostatic regulation
325 loop between contracting skeletal muscles and adipose tissue. Our data suggest that GDF15 is

326 rapidly produced upon skeletal muscle contraction and exercise mimetics. Unlike in mice,
327 GFRAL/RET expression is not highly specific to the caudal brain and displays a significant
328 expression in human adipose tissue. As GDF15 has emerged as an interesting anti-obesity
329 therapy through its central effects on appetite suppression, potential peripheral effects should
330 not be neglected. Hence, future studies of the effects of GDF15 in humans are highly needed.

331 **Methods**

332 **GDF15 protein level determination**

333 GDF15 protein levels in cultured CM and plasma samples were determined by ELISA (R&D
334 Systems, Minneapolis, USA).

335

336 **Lipolysis assay**

337 Whole cell lipolysis in differentiated hMADS adipocytes was assessed in control and EPS
338 CM supplemented with 2% BSA. The medium was changed, and following 3h of treatment,
339 glycerol and NEFAs released in the medium were measured using commercially available kits
340 (free glycerol reagent from Sigma and NEFA-C from Wako Chemicals). Data were
341 normalized to total cell protein content.

342 Thirty grams of subcutaneous abdominal adipose tissue were cut with scissors under aseptic
343 conditions into small pieces weighing approximately 10 mg or less. In all experiments, pieces
344 were washed three times with sterile PBS to remove blood cells. The pieces of adipose tissue
345 were centrifuged for 1 min at 300×g at room temperature between each wash. Then 20 g of
346 the pieces of tissue was resuspended in 50 ml of fresh medium containing 10% FCS. The
347 culture medium used was DMEM F12 (Cambrex, Verviers, Belgium) containing 33 μmol/l
348 biotin, 17 μmol/l pantothenate and 50 μg/ml gentamicin. The pieces (200mg/ml) were
349 incubated for 4h with recombinant human GDF15 concentrations of 1 and 100 ng/ml and
350 forskolin 10 μM as positive control. Aliquots of the medium were taken after 4h of incubation
351 and stored at -80°C for glycerol and NEFAs measurements as described above. The pieces
352 were washed three times with PBS, drained, and weighed for data normalization.

353

354 **Adipose Tissue Fractionation**

355 Briefly, after collagenase digestion (250 U/mL in phosphate-buffered saline (PBS), 2%
356 bovine serum albumin (BSA), pH 7.4, volume/volume) of the adipose tissue for 20 min at
357 37°C, the floating mature adipocytes were separated from the SVF. Then, the different SVF
358 cells were isolated using an immunoselection/depletion approach utilizing magnetic
359 microbeads coupled to specific CD antibodies (CD31, CD34, CD14) which are membrane
360 cell markers to select the different SVF cell types.

361 The preadipocytes are CD34-positive cells (CD34+) and CD31-negative (CD31-) cells. The
362 CD34-negative cells (CD34-) are immune cells (macrophages which are also CD14+ cells
363 and lymphocytes which are CD14- cells) as described in (47).

364

365 **Skeletal muscle cell primary culture**

366 Satellite cells from *rectus abdominis* of healthy male subjects (age 34.3 ± 2.5 years, BMI 26.0
367 ± 1.4 kg/m², fasting glucose 5.0 ± 0.2 mM) were kindly provided by Prof. Arild C. Rustan
368 (Oslo University, Norway). Satellite cells were isolated by trypsin digestion, preplated on an
369 uncoated petri dish for 1 h to remove fibroblasts, and subsequently transferred to T-25
370 collagen-coated flasks in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/L)
371 supplemented with 16% FBS and various factors (human epidermal growth factor, BSA,
372 dexamethasone, gentamycin, fungizone, fetuin) as previously described (48). Cells from
373 several donors were pooled and grown at 37 °C in a humidified atmosphere of 5% CO₂.
374 Differentiation of myoblasts (i.e. activated satellite cells) into myotubes was initiated at ~ 80–
375 90% confluence, by switching to α -Minimum Essential Medium with 2% penicillin-
376 streptomycin, 2% FBS, and fetuin (0.5 mg/ml). The medium was changed every other day and
377 cells were grown up to 5 days.

378

379 **Electrical Pulse Stimulation**

380 Skeletal muscle cells were fully differentiated for 4 days, and EPS was then applied using a
381 C-Pace EP multichannel culture pacer (IonOptix, Dublin, Ireland) for 3h (acute intense
382 exercise model) with a protocol consisting of 24 ms pulses at 10 V, with a frequency of 0,5
383 Hz, or for 24h (chronic moderate exercise model) with a protocol consisting of 2 ms pulses at
384 10 V, with a frequency of 0,1 Hz. Respective control media from unstimulated skeletal
385 muscle cells were obtained at 3h and 24h in paired cultures. After completion of EPS,
386 conditioned media (CM) were collected for proteomic screening and CM experiments with
387 hMADS adipocytes. The EPS protocol did not induce any visible cell detachment, and
388 assessment of the cytotoxic effect of EPS based on the release of adenylate kinase in culture
389 media using a colorimetric cytotoxicity assay (ToxiLight™ Non-Destructive Cytotoxicity
390 BioAssay Kit, Lonza, USA) showed that adenylate kinase activity was not significantly
391 changed in medium from stimulated compared to unstimulated myotubes.

392

393 **Culture of human multipotent adipose-derived stem cells**

394 hMADS cells were cultured and maintained in proliferation medium (DMEM low
395 glucose 1g/l, 10% FBS, 2 mM L-glutamine, 10 mM HEPES buffer, 50 units/ml of
396 penicillin, 50 mg/ml of streptomycin, supplemented with 2.5 ng/ml of human fibroblast
397 growth factor 2 (FGF2)) as previously described (49). The cells were inoculated in 6-well
398 plates at a density of 44'000 cells/ml and kept at 37°C in 5% CO₂. Six days post-seeding,
399 FGF2 was removed from proliferation medium. On the next day (day 0), the cells were
400 incubated in differentiation medium (DM; serum-free proliferation medium/Ham's F-12
401 medium containing 10 µg/ml of transferrin, 10 nM of insulin, 0.2 nM triiodothyronine, 100

402 μM 3-isobutyl-1- methylxanthine, 1 μM dexamethasone and 100 nM rosiglitazone). At day 3,
403 dexamethasone and 3-isobutyl-1-methylxanthine were omitted from DM and at day 10
404 rosiglitazone was also omitted. Lipolysis experiments with EPS CM and the GDF15
405 neutralizing antibody were carried out at day 14. Human FGF2, insulin, triiodothyronine,
406 transferrin, 3-isobutyl-1-methylxanthine, and dexamethasone were from Sigma; L-glutamine,
407 penicillin, and streptomycin from Invitrogen; HEPES, Dulbecco's modified Eagle medium low
408 glucose, and Ham's F-12 medium from Lonza; and rosiglitazone from Alexis Biochemicals.

409

410 **Determination of glucose metabolism**

411 Total glycogen content was determined spectrophotometrically after complete hydrolysis into
412 glucose by the α -amylglucosidase as previously described (50). For glycogen synthesis
413 experiments, myotubes were pre-incubated with a glucose- and serum-free medium for 90
414 min, then exposed to DMEM supplemented with D[U- ^{14}C] glucose (1 $\mu\text{Ci}/\text{ml}$; PerkinElmer,
415 Boston, MA) for 3 hours. Following incubation, glucose oxidation was determined by
416 counting of $^{14}\text{CO}_2$ released into the culture medium. The cells were then solubilized in KOH
417 30% and glycogen synthesis was determined as previously described (26).

418

419 **Determination of palmitate oxidation**

420 Myotubes were preincubated for 3 hours with [1- ^{14}C]palmitate (1 $\mu\text{Ci}/\text{mL}$; PerkinElmer) and
421 nonlabeled (cold) palmitate (100 μM final concentration). Palmitate was coupled to a fatty
422 acid (FA)-free BSA in a molar ratio of 5:1. After incubation, $^{14}\text{CO}_2$ and ^{14}C -acid soluble
423 metabolite were measured as previously described (51). All assays were performed in
424 duplicate, and data were normalized to cell protein content.

425

426 **Mass spectrometry-based quantitative analysis of myotube secretome**

427 Methods are detailed in Supplementary Methods. After protein extraction from culture media
428 (N=4 per group), one sample pool, comprising equal amounts of all protein extracts, was
429 generated for quality assessment of LC-MS/MS. Individual samples and the sample pool were
430 electrophoresed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Five
431 protein bands (2mm each) per lane were excised from the gels, and proteins were in-gel
432 reduced and alkylated using an automatic pipetting device (MassPrep, Waters), then digested
433 at 37°C overnight with trypsin (Promega, Madison, WI, USA). A set of reference peptides
434 [Indexed Retention Time (iRT) Kit; Biognosys, Schlieren, Switzerland] was added to the
435 resulting peptides to allow the stability of instrument performances to be measured for QC
436 purposes.

437 Samples were analyzed on a nano-ultraperformance LC system (nanoAcquity; Waters,
438 Milford, MA, USA) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive
439 plus; ThermoFisher Scientific). Mass spectrometry data were processed using MaxQuant
440 software (v.1.5.8.3; Max Planck Institute of Biochemistry, Martinsried, Germany) (52). Peak
441 lists were created using default parameters and searched using the Andromeda search engine
442 (revert mode) implemented in MaxQuant against a protein database created using the MSDA
443 software suite (53). The database contained human protein sequences (Swiss-
444 Prot; <https://www.uniprot.org/taxonomy/>; Taxonomy ID: 9606; 20,195 entries), which were
445 downloaded in October 2017. Sequences of common contaminants like keratins and trypsin
446 (247 entries) were added to the database (contaminants.fasta included in MaxQuant). A false
447 discovery rate of 1% for both peptide spectrum matches (minimum length of 7 amino acids)
448 and proteins was accepted during identification. Regarding quantification, data normalization

449 and protein abundance estimation was performed using the label-free quantification (LFQ;
450 (52)) option offered in MaxQuant. Proteins identified with only one unique peptide were not
451 considered for quantification. Only proteins with at least three of four valid values per group
452 as well as the ones absent (i.e., 0 valid values) in samples from a given group were kept for
453 further analysis. The MS proteomics data have been deposited to the ProteomeXchange
454 Consortium via the Pride partner repository with the dataset identifier PXD014126.

455 QC-related measurements showed that HPLC performance remained good and stable
456 throughout the whole experiment, with a median coefficient of variation of 0.5% concerning
457 retention times of all iRT peptides when considering all injections. The reproducibility of
458 quantitative data was also satisfactory because we recorded low median coefficients of
459 variation for MaxQuant-derived LFQ values of all quantified proteins within each of the four
460 experimental groups (31%) and in the sample pool of all samples injected repeatedly during
461 the course of MS-based analyses (21%).

462

463 **Real-time qPCR**

464 Total RNA from cultured myotubes or *vastus lateralis* muscle was isolated using Qiagen
465 RNeasy mini kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany).
466 The quantity of RNA was determined on a Nanodrop ND-1000 (Thermo Scientific, Rockford,
467 IL, USA). Reverse-transcriptase PCR was performed on a Techne PCR System TC-412 using
468 the Multiscribe Reverse Transcriptase method (Applied Biosystems, Foster City, CA). Real-
469 time quantitative PCR (qPCR) was performed to determine cDNA content. All primers were
470 bought from Applied Biosystems and were: *18S* (Taqman assay ID: Hs99999901_s1), *GDF15*
471 (Hs00171132_m1), *GFRAL* (Hs01087628_m1), *RET* (Hs01120030_m1), *IL6*
472 (Hs00985639_m1), *IL15* (Hs01003716_m1), *FGF21* (Hs00173927_m1), *Myostatin*

473 (Hs00976237_m1), *FNDC5* (Hs00401006_m1), *Apelin* (Hs00175572_m1), *BDNF*
474 (Hs03805848_m1). The amplification reaction was performed in duplicate on 20ng of cDNA
475 in 96-well reaction plates on a StepOnePlus™ system (Applied Biosystems). All expression
476 data were normalized by the $2^{-\Delta Ct}$ method using *18S* or *PUM1* as internal controls for cell
477 type or whole adipose tissue data, respectively.

478

479 **Statistics**

480 All statistical analyses were performed using GraphPad Prism 7.0 for Windows (GraphPad
481 Software Inc., San Diego, CA). Normal distribution and homogeneity of variance of the data
482 were tested using Shapiro-Wilk and F tests, respectively. Two-tailed Student's unpaired and
483 paired t-tests were performed to determine differences between treatments. Two-way
484 ANOVA and Bonferroni's post hoc tests were used when appropriate. All values in figures
485 and tables are presented as mean \pm SEM. Statistical significance was set at $p \leq 0.05$.

486

487 **Supplemental Information**

488 The supplemental information contains 5 figures, supplemental methods and references.

489

490 **Author contributions**

491 CL, AP, EM, DC, BL, PM, JV, NB, CF, MM, DL, IH, CT, EM, SCB, VB, GT, JLG, AB, DL,
492 NV, FB, SB, IdG, DOG, and CM researched data and edited the manuscript, CL, DOG, FB,
493 and CM contributed to study design and revised the manuscript, CL and CM wrote the
494 manuscript.

495

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654 **Figures Legend**

655 **Figure 1. Acute intense and chronic moderate exercise model validation in human**
656 **myotubes**

657 (A-D) Acute intense exercise model, EPS3h: (A) total glycogen content, (B) lactate secretion,
658 (C) glucose oxidation rate, and (D) palmitate oxidation rate in five days cultured human
659 myotubes submitted to 3h EPS to mimic an acute high intensity exercise *in vitro*. Data are
660 expressed as mean \pm SEM (n=8). *p < 0.05, **p < 0.01, ***p < 0.001 compared to control by
661 two-tailed unpaired Student t-test.

662 (E-H) Chronic moderate exercise model, EPS24h: (E) total glycogen content, (F) basal
663 glycogen synthesis rate, (G) glucose oxidation rate, and (H) palmitate oxidation rate in five
664 days cultured human myotubes submitted to 24h EPS to mimic moderate chronic exercise
665 training *in vitro*. Data are expressed as mean \pm SEM (n=8). *p < 0.05 compared to control by
666 two-tailed unpaired Student t-test.

667 (I-K) Conditioned media experiments between human myotubes and human adipocytes: (I)
668 experimental design, (J) conditioned media of EPS3h-stimulated myotubes, and (K)
669 conditioned media of EPS24h-stimulated myotubes were applied on differentiated hMADS
670 adipocytes for 24h to measure glycerol output, a surrogate of lipolysis. Data are expressed as
671 mean \pm SEM (n=16-20). ***p < 0.01 compared to control by two-tailed unpaired Student t-
672 test.

673

674 **Figure 2. Identification of GDF15 as a novel exercise-regulated myokine**

675 (A-C) Proteomic screen of conditioned media from EPS3h-stimulated myotubes: (A) Volcano
676 plot of the unbiased proteomic screen reveal GDF15 as a significantly up-regulated protein in

677 conditioned media, (B) quantification of GDF15 protein in media from the protein screen, (C)
678 quantification of GDF15 protein secretion by ELISA in five days cultured human myotubes
679 submitted to 3h EPS to mimic an acute intense exercise *in vitro*. Data are expressed as mean \pm
680 SEM (n=16). *p < 0.05, **p < 0.01 compared to control by two-tailed paired Student t-test.

681 (D-F) Proteomic screen of conditioned media from EPS24h-stimulated myotubes: (D)
682 Volcano plot of the unbiased proteomic screen reveal GDF15 as a significantly up-regulated
683 protein in conditioned media, (E) quantification of GDF15 protein in media from the protein
684 screen, (F) quantification of GDF15 protein secretion by ELISA in five days cultured human
685 myotubes submitted to 24h EPS to mimic a moderate chronic exercise training *in vitro*. Data
686 are expressed as mean \pm SEM (n=16). *p < 0.05 compared to control by two-tailed paired
687 Student t-test.

688 (G) Time-course of GDF15 protein secretion in acutely EPS-stimulated myotubes. Data are
689 expressed as mean \pm SEM (n=6). **p < 0.01 compared to control by two-way ANOVA
690 followed by a Bonferroni post-hoc test.

691

692 **Figure 3. GDF15 gene expression changes in contracting skeletal muscle**

693 (A) Time-course of GDF15 mRNA level in acutely EPS-stimulated myotubes. Data are
694 expressed as mean \pm SEM (n=6). **p < 0.01, ***p < 0.001 compared to control by two-way
695 ANOVA followed by a Bonferroni post-hoc test.

696 (B) GDF15 mRNA level in EPS3h-stimulated myotubes and (C) in EPS24h-stimulated
697 myotubes. Data are expressed as mean \pm SEM (n=12). **p < 0.01 compared to control by
698 two-tailed unpaired Student t-test.

699 (D-F) GDF15 mRNA level changes in response to exercise-mimetics: five days cultured
700 human myotubes were treated for 3h with (D) Forskolin 10 μ M, (E) Ionomycin 4 μ M, (F) and
701 the PPAR δ agonist GW0742 100 nM. Data are expressed as mean \pm SEM (n=6). **p < 0.01,
702 ***p < 0.001 compared to control by two-tailed unpaired Student t-test.

703 (G) GDF15 mRNA level changes in muscle *vastus lateralis* biopsy samples taken before and
704 immediately after a 1h acute exercise bout in lean healthy volunteers (Human study 1). Data
705 are expressed as mean \pm SEM (n=18). ***p < 0.001 compared to control by two-tailed paired
706 Student t-test.

707

708 **Figure 4. Changes in plasma GDF15 levels with exercise, obesity and lifestyle**
709 **intervention**

710 (A-C) Human study 2: plasma GDF15 changes in lean healthy volunteers in response to (A)
711 endurance exercise 1h at 60% VO₂max, (B) sprint interval exercise 7 repetitions at 130% of
712 maximal workload, (C) delta change from rest to exercise during endurance versus sprint
713 interval exercise. Data are expressed as mean \pm SEM (n=8-15). **p < 0.01 compared to
714 control by two-tailed paired Student t-test.

715 (D-F) Human study 3: plasma GDF15 changes in obese middle-aged (D) and elderly (E)
716 subjects at baseline and in response to a 8-week lifestyle intervention-induced weight loss
717 program. Data are expressed as mean \pm SEM (n=8). **p < 0.01, ***p < 0.001 compared to
718 control by two-tailed paired Student t-test. (F) Plasma GDF15 changes in response to
719 lifestyle-induced weight loss in obese individuals (all subjects). Data are expressed as mean \pm
720 SEM (n=16). *p < 0.05 compared to control by two-tailed paired Student t-test.

721 (G) Plasma GDF15 levels in lean healthy (study 2) versus middle-aged obese (study 3).

722 (H) Plasma GDF15 levels in middle-aged obese versus elderly obese (study 3).

723

724 **Figure 5. GDF15 promotes lipolysis in human adipose tissue**

725 (A-B) Lipolytic response measured by glycerol release (A) and non-esterified fatty acid
726 release (B) in human abdominal subcutaneous adipose tissue explants in basal condition and
727 in response to 1 and 100 ng/ml of recombinant human GDF15, 10 μ M forskolin (FK) and 1
728 μ M isoprenaline (ISO). Data are expressed as mean \pm SEM (n=18, 3 replicates of 6
729 independent donors). *p < 0.05, **p < 0.01 compared by unpaired Student t-test.

730 (C-D) Lipolytic response measured by glycerol release (C) and non-esterified fatty acid
731 release (D) in human abdominal subcutaneous adipose tissue explants in basal condition and
732 in response to 100 ng/ml of recombinant human GDF15. Data are expressed as mean \pm SEM
733 (n=6 independent donors). *p < 0.05 compared by unpaired Student t-test.

734 (E) Conditioned media of EPS24h-stimulated myotubes were applied on differentiated
735 hMADS adipocytes for 24h to measure glycerol output, a surrogate of lipolysis, in absence or
736 presence of a GDF15 neutralizing antibody. Data are expressed as mean \pm SEM (n=5). *p <
737 0.05 compared to control by one-way ANOVA.

738 (F) Integrative model of the skeletal muscle-adipose tissue crosstalk orchestrated by GDF15.
739 Upon muscle contraction, GDF15 is released, reach its target receptors GFRAL/RET on
740 adipose tissue to activate lipolysis which releases non esterified fatty acid further serving as
741 fuels to supply muscle contraction.

742 **Table 1. Relationship between biological and anthropometric variables and**
 743 **subcutaneous abdominal adipose tissue GFRAL and RET mRNA levels**

Biological variable	Gene Symbol	r	P-value	n
Triglycerides	<i>GFRAL</i>	0.126	0.022	329
	<i>RET</i>	0.178	0.001	326
Total cholesterol	<i>GFRAL</i>	0.056	0.308	332
	<i>RET</i>	0.114	0.034	329
Glucose	<i>GFRAL</i>	0.112	0.046	316
	<i>RET</i>	0.085	0.135	313
HOMA-IR	<i>GFRAL</i>	0.137	0.017	306
	<i>RET</i>	0.157	0.006	303
Matsuda index	<i>GFRAL</i>	-0.127	0.027	305
	<i>RET</i>	-0.166	0.004	302
BMI	<i>GFRAL</i>	0.028	0.693	330
	<i>RET</i>	-0.121	0.029	327
Waist circumference	<i>GFRAL</i>	0.106	0.053	329
	<i>RET</i>	0.041	0.460	326

744 These data were obtained from Human study 4. BMI, body mass index; HOMA-IR,
 745 homeostatic model assessment of insulin resistance; r: Pearson correlation coefficients. The
 746 Matsuda index was calculated as $10\,000/\text{square root of } [(fasting\ glucose \times fasting\ insulin) \times$
 747 $(mean\ glucose \times mean\ insulin\ during\ OGTT)]$.