Supplemental Information for:

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

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**Supplemental Figure 1. Activation of exercise-signaling pathways and myokine gene expression by EPS in human myotubes**

(A) Representative blot of p38 MAPK and CaMKII phosphorylation in five days cultured human myotubes submitted to a time-course of EPS up to 48h.

(B) Canonical myokines mRNA level in EPS3h-stimulated myotubes. Data are expressed as mean ± SEM (n=12). *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (CON) by two-way ANOVA.

(C) Canonical myokines mRNA level in EPS24h-stimulated myotubes. Data are expressed as mean ± SEM (n=12). *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (CON) by two-way ANOVA.

**Supplemental Figure 2. Metabolic effects of EPS24h**

(A) Effect of EPS24h on lactate production in human myotubes (n=12).

(B) Influence of conditioned media from EPS24h-stimulated myotubes on NEFA release in human adipocytes (n=5).

**Supplemental Figure 3. Relationship between GDF15, systemic lipolysis and weight loss**

(A) Correlation between plasma level of GDF15 and plasma glycerol levels in obese subjects at rest and during exercise (45 min at 50% VO2max) (Human study 3, n=47).

(B) Correlation between changes in plasma GDF15 levels during very low calorie diet (VLCD)-induced weight loss in obese individuals and changes in body mass index (Human study 4, n=28).
Supplemental Figure 4. GDF15 receptors expression in subcutaneous abdominal adipose tissue as a function of adiposity

(A-B) Relative gene expression of GFRAL (A) and relative gene expression of RET (B) in human whole abdominal subcutaneous adipose tissue, isolated adipocytes and stroma vascular fraction (SVF) preparations from the same samples (Human study 4), and in whole human skeletal muscle tissue. Data are expressed as mean ± SEM (n=5-7).

(C-D) Relative gene expression of GFRAL (C) and RET (D) in abdominal subcutaneous adipose tissue sorted stromal cells, specifically preadipocytes, lymphocytes and macrophages (n=5).

(E-F) Relative gene expression of GFRAL (E) and its co-receptor RET (F) in subcutaneous abdominal adipose tissue samples in a large cohort of Caucasians individuals as a function of adiposity and obesity grade (Human study 4). Data are expressed as mean ± SEM (n=326).

Supplemental Figure 5. GDF15 receptors expression in murine tissues

(A-B) Relative gene expression of GDF15 (A) and GFRAL (B) in whole brain, perigonadic white adipose tissue and skeletal muscle vastus lateralis of adult female mice. Data are expressed as mean ± SEM (n=20).
Supplementary Methods

Human Study 1
Young recreationally active (age 22.1±3.5 years; BMI 24.0±1.6 kg.m\(^{-2}\); VO\(_{2\text{max}}\) 48.5±3.7 ml.kg\(^{-1}\).min\(^{-1}\)) males recruited to partake in this study. The protocol was approved by the Ethics Committee of Dublin City University and all participants gave written informed consent. VO\(_{2\text{peak}}\) was determined on a cycle ergometer starting at 70 watts and increasing in 30-watt increments every 3 minutes until exhaustion. At least 7 days later participants completed a 60 min bout of exercise on an electrically braked cycle ergometer at 55% peak power output. A skeletal muscle biopsy of the vastus lateralis was performed pre and immediately post the exercise bout. Muscle samples were immediately frozen in liquid nitrogen before being stored at -80 °C.

Human study 2
This was a cross-sectional study comparing an acute bout of moderate continuous exercise with a high intensity interval bout of exercise in young active men (VO\(_{2\text{peak}}\) 50-65 ml.kg\(^{-1}\).min\(^{-1}\)). The protocol was approved by the Dublin City University Ethics Committee and all subjects gave written informed consent. Participants were instructed to refrain from exercise and to replicate food intake the day before each trial. In the morning, following an overnight fast, participants lay on a bed for 1-hr after arriving at the lab. A blood sample was taken and they then exercised (i) on a bicycle ergometer at 60% VO\(_{2\text{peak}}\) for 1-hr or (ii) by performing 7 high intensity bouts of exercise at 130% peak power output. Each bout lasted 30-sec followed by 4.5-min recovery. A blood sample was taken at the end of each exercise trial. The intensity for both trials was determined using the results of an incremental exercise test to exhaustion.
**Human Study 3**

Middle-aged (age 36.5±0.6 years; BMI 34.2±0.8 kg.m\(^2\)) and elderly (age 64.2±0.9 years; BMI 33.9±0.4 kg.m\(^2\)) obese male subjects were enrolled in the MONA (Metabolism, Obesity, Nutrition and Age) clinical trial NCT02161926. The protocol was approved by the Ethics Committee of Toulouse University Hospitals, and all subjects gave written informed consent. They participated in an 8-week lifestyle intervention including a moderate calorie restriction of 20% below their daily energy requirement aerobic combined to two sessions of resistance exercise per week. The participants were asked to refrain from vigorous physical activity 48 hours before presenting to the clinical investigation center, and they ate a weight-maintaining diet consisting of 35% fat, 15% protein, and 50% carbohydrates 2 days before the experiment. Maximal oxygen uptake (VO2max) was investigated on a bicycle ergometer by indirect calorimetry at baseline. Anthropometric and blood parameters were assessed at rest and during a 45 min acute exercise bout performed at 50% VO2max, at baseline and 48-72h after the end of the lifestyle intervention. Blood was collected on EDTA and immediately processed for plasma storage at -80°C.

**Human Study 4**

The samples investigated in this paper were collected from 2006 to 2007 during the DiOGenes study, a pan-European randomized trial, which was approved by the ethics committees of each of the 8 European centers participating to the program (NCT00390637). The DiOGenes project investigated the effects of diets with different content of protein and glycemic index on weight-loss maintenance and metabolic and cardiovascular risk factors after an 8-week very low calorie diet (VLCD) phase, in obese/overweight individuals. Written informed consent was obtained from each patient according to the local ethics committee of the participating countries as previously described (1).
Healthy overweight (body mass index (BMI) \( \geq 27 \text{kg/m}^2 \)) individuals, aged <65 years were eligible for the study. Exclusion criteria were BMI >45 kg/m2, liver or kidney diseases, cardiovascular diseases, diabetes mellitus type 1, special diets/eating disorders, systemic infections/chronic diseases, cancer within the last 10 years, weight change >3 kg within the previous 3 months, and other clinical disorders or use of prescription medication that might interfere with the outcome of the study.

A detailed description of inclusion and exclusion criteria has been published previously (1). A detailed description of the DiOGenes intervention trial and main outcomes can be found in the core publication (1). Briefly, among 1209 individuals screened, 932 entered a baseline clinical investigation day including anthropometric measures (height, weight, waist circumference, body composition), blood pressure measurements, fasting blood sampling, and subcutaneous adipose tissue biopsies were performed (at baseline and at the end of each phase). All procedures were standardized between the 8 study centers across Europe. Only baseline and VLCD biological samples and clinical data were used in the present investigation. Paired adipose tissue RNA samples were available at baseline and at the end of the VLCD for 359 individuals (age 42.6±6.3 years; BMI 34.1±4.6 kg.m\(^{-2}\)). Plasma GDF15 was investigated in a subset of 28 individuals (11 men and 17 women) with both clinical and RT-qPCR data available at both baseline and after VLCD.

Mass spectrometry-based quantitative analysis of myotube secretome

Culture media (N=4 per group) were first supplemented with 0.1% of sodium lauroyl sarcosinate (Sigma-Aldrich, St. Louis, MO, USA) and 7.5% of trichloroacetic acid (TCA; Sigma-Aldrich), then centrifuged (5400g, 20 minutes, 4°C). Supernatants were discarded and protein pellets were washed twice, using 2ml of cold tetrahydrofuran (THF; VWR International S.A.S, Fontenay-sous-Bois, France) followed by centrifugation (5400g, 20
minutes, 4°C, then 15000g, 15 minutes, 4°C) and elimination of supernatants. Proteins were then solubilized in a solution composed of 5% of sodium dodecyl sulfate (SDS; Thermo Fisher Scientific, Waltham, MA, USA), 10mM of Tris pH 6.8 (Sigma-Aldrich), 1mM of ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), and 10% of glycerol (Sigma-Aldrich). Protein concentration was measured using the Bradford protocol (Bio-Rad, Hercules, CA, USA). At this stage, one sample pool, comprising equal amounts of all protein extracts, was generated for quality assessment of LC-MS/MS.

Dithiothreitol (DTT; 50mM; Sigma-Aldrich) and bromophenol blue (0.1%; Sigma-Aldrich) were added to aliquots of 10 µg of proteins (individual samples and sample pool). After incubation at 95°C for 5 minutes, proteins were electrophoresed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels composed of (4% stacking (4% acrylamide; Sigma-Aldrich) and a resolving gel (12% acrylamide). After incubation in a solution composed of ethanol (50%) and phosphoric acid (3%), followed by 4 washing steps in water, staining was performed using colloidal Coomassie blue (Sigma-Aldrich). Five protein bands (2mm each) per lane were excised from the gels. Proteins were in-gel reduced and alkylated using an automatic pipetting device (MassPrep, Waters) and digested at 37°C overnight with trypsin (Promega, Madison, WI, USA) at an enzyme-to-protein-ratio of 1:50. Peptides were extracted twice in 60% acetonitrile (ThermoFisher Scientific)/0.1% formic acid (ThermoFisher Scientific) in water for 1h, then once in 100% acetonitrile at 450rpm on an orbital shaker. At this stage, a set of reference peptides [Indexed Retention Time (iRT) Kit; Biognosys, Schlieren, Switzerland] was added to each sample to allow the stability of instrument performances to be measured for QC purposes. Just before LC-MS/MS analysis, the volume of peptide extracts was reduced in a speed-vac and sample volumes were adjusted to 40 µl with 0.1% formic acid in water.
Samples were analyzed on a nano-ultraperformance LC system (nanoAcquity; Waters, Milford, MA, USA) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus; ThermoFisher Scientific). Six microliters of each sample were concentrated/desalted on a trap column (Symmetry C18, 180 µm x 20 mm, 5 µm; Waters) using 99% of solvent A (0.1% formic acid in water) and 1% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 5 µl/minute for 3 minutes. Afterwards, peptides were transferred and eluted from the separation column (BEH 130 C18, 75 µm x 250 mm, 1.7 µm; Waters) maintained at 60°C using a 80-minute gradient from 1 to 35% of solvent B at a flow rate of 450 nl/minute. All samples were injected using a randomized and blocked injection sequence (1 biologic replicate of each group plus the pool in each block). To minimize carryover, a column wash (50% ACN for 20 min) was included between each block in addition to a solvent blank injection, which was performed after each sample. The Q Exactive Plus instrument was operated in positive ion mode with source temperature set to 250°C and spray voltage set to 1.8 kV. Full scan MS spectra [mass-to-charge ratio (m/z) 300–1800] were acquired at a resolution of 140,000 at m/z 200, a maximum injection time of 50 ms, and an automatic gain control target value of 3x10^6 charges with the lock-mass option enabled (m/z 445.12002). The 10 most intense precursors per full scan were isolated using a m/z 2 window and fragmented using higher energy collisional dissociation (normalized collision energy of 27 eV). Dynamic exclusion of already-fragmented precursors was set to 60 s. MS/MS spectra were acquired with a resolution of 17,500 at m/z 200, a maximum injection time of 100 ms, and an automatic gain control target value of 1x10^5. The system was fully controlled by XCalibur software (v. 3.0.63; Thermo Fisher Scientific).

Mass spectrometry data were processed using MaxQuant software (v.1.5.8.3; Max Planck Institute of Biochemistry, Martinsried, Germany) (2). Peak lists were created using default parameters and searched using the Andromeda search engine (revert mode) implemented in
MaxQuant against a protein database created using the MSDA software suite (3). The database contained human protein sequences (Swiss-Prot; https://www.uniprot.org/taxonomy/; Taxonomy ID: 9606; 20,195 entries), which were downloaded in October 2017. Sequences of common contaminants like keratins and trypsin (247 entries) were added to the database (contaminants.fasta included in MaxQuant). The first search was performed using a precursor mass tolerance of 20 ppm and the main search with a tolerance of 5 ppm after recalibration. Fragment ion mass tolerance was set to 20 ppm. Carbamidomethylation of cysteine residues was considered as fixed and oxidation of methionine residues and acetylation of protein N termini as variable modifications during the search. A maximum number of 1 missed cleavages and a false discovery rate of 1% for both peptide spectrum matches (minimum length of 7 amino acids) and proteins was accepted during identification. Regarding quantification, data normalization and protein abundance estimation was performed using the label-free quantification (LFQ; (2)) option offered in MaxQuant using a minimal ratio count of one. “match between runs” was enabled using a 1.5-min time window after retention time alignment using a 20-min time window. Both unmodified and modified (acetylation of protein N termini and oxidation of methionine residues) peptides were considered for quantification whereas shared peptides were excluded. All other MaxQuant parameters were set as default. Proteins identified with only one unique peptide were not considered for quantification. Only proteins with at least three of four valid values per group as well as the ones absent (i.e., 0 valid values) in samples from a given group were kept for further analysis. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the Pride (24) partner repository with the dataset identifier PXD014126.

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

**Western blot**

Proteins were extracted from tissues using Ripa buffer and protease inhibitor cocktail (Sigma-Aldrich, #R0278 and #P8340/#P5726/#P0044). Tissues homogenates were centrifuged twice for 20 min at 12700 rpm and supernatants were quantified with BCA pierce kit (ThermoScientific, #23225). A total of 30 µg of proteins were run on a 4-20% SDS-polyacrylamide gel electrophoresis (Biorad), transferred onto nitrocellulose membrane (BioRad) and incubated overnight at 4°C with primary antibodies, Rabbit anti-P38MAPK (1/1000, CST, #9212), and Rabbit anti-CaMKII (1:1000, CST, #3362). Subsequently, immunoreactive proteins were blotted with anti-rabbit or goat horseradish peroxidase-labeled secondary antibodies for 1h at room temperature and revealed by enhanced chemiluminescence reagent (SuperSignal West Femto, Thermo Scientific), visualized using ChemiDoc MP Imaging System and data analyzed using the ImageLab 4.2 version software (Bio-Rad Laboratories, Hercules, USA).
Supplementary References


Supplemental Figure 1

A

Thr180 p38 MAPKα
p38 MAPKα
Thr286 pCaMKII
CaMKII

B

Relative gene expression

IL6 IL15 FGF21 Myostatin FNDC5 Apelin BDNF

C

Relative gene expression

IL6 IL15 FGF21 Myostatin FNDC5 Apelin BDNF
Supplemental Figure 2

A

B

Control EPS

Lactate release (fold change over control)

NEFA release (fold change over control)

Control EPS
Supplemental Figure 3

A

Plasma GDF15 (pg/ml) vs. Plasma glycerol (µmol/l)

B

BMI loss during VLCD vs. Change in plasma GDF15 during VLCD

$r=0.288$

$p=0.049$

$r=0.350$

$p=0.067$
Supplemental Figure 4

A

B

C

D

E

F

Relative gene expression

GFRAL

RET

Relative gene expression

GFRAL

RET

Relative gene expression

GFRAL

RET

Relative gene expression

GFRAL

RET

Relative gene expression

GFRAL

RET

Relative gene expression
Supplemental Figure 5

A

GDF15

Brain  EW AT  Muscle

Relative gene expression

B

GFRAL

Brain  EW AT  Muscle

Relative gene expression