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Professor Christos Mantzoros
Editor-in-chief
Metabolism, Clinical and Experimental

Barcelona, 2020-02-14

Dear Prof. Mantzoros,

Please find enclosed the revised version of the article **METABOLISM-D-19-00958 (R2)** entitled “**Low-density lipoprotein receptor-related protein 1 deficiency in cardiomyocytes reduces susceptibility to insulin resistance and obesity**” which we resubmit for your consideration for publication in *Metabolism, Clinical and Experimental*.

Following the request of Reviewer 1, we have been looking for a suitable ELISA system to measure AMPK activity in frozen tissues. There are not many options that meet this condition except the ELISA with the reference CY-1182 from BioNova. We have performed this ELISA assay with hepatic tissue from the four study II groups. Positive and negative controls were in the optimal range [positive control=1.33±0.05 (optimal>1), and negative control=0.13±0.00 (optimal<0.25)]. However, we did not detect significant differences in the hepatic AMPK kinase activity between the study groups and, in particular, between *cm-Lrp1*^{-/-} compared to control group (2.30±0.19 versus 1.91±0.25; *P*=0.395). These results have been included in a new Figure sent in the the Answers to Reviewers file. According to the manufacturer, this kit is designed to evaluate AMPK activators/inhibitors and AMPK activity of purified enzyme. Although the possibility of using cells/tissue is also offered in the kit, the manufacturer warns about the possibility of detecting other kinase besides AMPK activities. In fact, the validation of AMPK activity by Western blot analysis is highly recommended. Dorsomorphin or compound C, the inhibitor advised to use in the ELISA, has been reported to inhibit other kinases much more potently than AMPK and therefore to be a non-specific inhibitor (Dasgupta B & Seibel W. *Methods Mol Biol* 2018; 1732: 195-202). We regret not having currently a system allowing a reliable detection of AMPK activity other than Western blot.

In this work, Western blot analysis showed an increased ratio of pAMPK/total AMPK and, importantly, of pACC/total ACC, the downstream target of pAMPK, in the liver of untreated *cm-Lrp1*^{-/-} compared to the control mice (Figure 5A). This increase in the phosphorylation of AMPK and ACC was not observed in A91915-treated mice, supporting that ANP signaling activates this AMPK-related energy sensing pathway in the liver of *cm-Lrp1*^{-/-} mice.

We hope that the manuscript will be acceptable for publication in *Metabolism, Clinical and Experimental*.

All authors have read and approved submission of the manuscript and the manuscript has not been published and is not being considered for publication elsewhere in whole or part in any language except as an abstract. None of the authors have any conflict of interest with the publication of the manuscript.

We thank you for your time and effort.

Sincerely

Dr. Vicenta Llorente Cortés

HIGHLIGHTS

- Cardiomyocyte *Lrp1* deficiency preserves corin activity and promotes ANP release
- Increased blood ANP levels activate cGMP/pVASP axis in skeletal muscle and liver
- In the liver, but not in skeletal muscle, cGMP/pVASP axis was linked to AMPK phosphorylation and activation
- Increased hepatic pAMPK promotes whole-body systemic energy expenditure

Low-density lipoprotein receptor-related protein 1 deficiency in cardiomyocytes reduces susceptibility to insulin resistance and obesity

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Abstract

Background

Low-density lipoprotein receptor-related protein 1 (LRP1) plays a key role in fatty acid metabolism and glucose homeostasis. In the context of dyslipemia, LRP1 is upregulated in the heart. Our aim was to evaluate the impact of cardiomyocyte LRP1 deficiency on high fat diet (HFD)-induced cardiac and metabolic alterations, and to explore the potential mechanisms involved.

Methods

We used *TnT-iCre* transgenic mice with thoroughly tested suitability to delete genes exclusively in cardiomyocytes to generate an experimental mouse model with conditional *Lrp1* deficiency in cardiomyocytes (*TnT-iCre*⁺-LRP1^{fl/fl}).

Findings

Mice with *Lrp1*-deficient cardiomyocytes (*cm-Lrp1*^{-/-}) have a normal cardiac function combined with a favorable metabolic phenotype against HFD-induced glucose intolerance and obesity. Glucose intolerance protection was linked to higher hepatic fatty acid oxidation (FAO), lower liver steatosis and increased whole-body energy expenditure. Proteomic studies of the heart revealed decreased levels of cardiac pro-atrial natriuretic peptide (pro-ANP), which was parallel to higher ANP circulating levels. *cm-Lrp1*^{-/-} mice showed ANP signaling activation that was linked to increased fatty acid (FA) uptake and increased AMPK/ ACC phosphorylation in the liver. Natriuretic peptide receptor A (NPR-A) antagonist completely abolished ANP signaling and metabolic protection in *cm-Lrp1*^{-/-} mice.

Conclusions

These results indicate that an ANP-dependent axis controlled by cardiac LRP1 levels modulates AMPK activity in the liver, energy homeostasis and whole-body metabolism.

Key Words: Cardiomyocyte, *Lrp1*, ANP, heart, liver, AMPK, fatty acid oxidation

Nonstandard Abbreviations and Acronyms

1	ACC	Acetyl-CoA carboxylase
2	Akt	Protein Kinase B
3	AMPK	AMP-activated protein kinase
4	ANP	Atrial natriuretic peptide
5	AUC	Area under the curve
6	BAT	Brown adipose tissue
7	BSA	Phosphate buffered saline
8	BNP	Brain natriuretic peptide
9	CE	Cholesteryl esters
10	cGMP	Cyclic guanosine monophosphate
11	CLAMS	Comprehensive lab animal monitoring system
12	CPT1	Carnitine palmitoyltransferase I
13	DAPI	4',6-diamidino-2-phenylindole
14	eWAT	Epididymal fat
15	FA	Fatty acid
16	FAO	Fatty acid oxidation
17	FASP	Filter Aided Sample Preparation
18	FC	Free cholesterol
19	FFA	Free fatty acid
20	GDF15	Growth and differentiation factor 15
21	GLUT	Glucose transporter
22	GTT	Glucose tolerance test
23	HDL	High density lipoprotein
24	HF	Heart failure
25	HFD	High fat diet
26	IR	Insulin resistance
27	LDLR	Low density lipoprotein receptor
28	LIPG	Endothelial lipase
29	LpL	Lipoprotein lipase
30	LRP1	Low-density lipoprotein receptor-related protein 1
31	LV	left ventricle
32	LVAWd	LV anterior wall at end cardiac diastole
33	LVEDV	LV end-diastolic volume
34	LVEF	LV ejection fraction
35	LVESV	LV end-systolic volume
36	LVFS	LV fractional shortening

1	LVIDd	LV internal diameter diastole
2	LVIDs	LV internal diameter systole
3	LVPWd	LV posterior wall at end cardiac diastole
4	NP	Natriuretic peptide
5	NPR-A	Natriuretic peptide receptor A
6	NPR-C	Natriuretic peptide receptor C
7	OFG	Oral fat gavage
8		Mitochondrial Oxidative Phosphorylation System
9	pAMPK	Phosphorylated AMP-activated protein kinase
10	pNpp	p-nitrophenyl phosphate
11	Pro-ANP	Pro-atrial natriuretic peptide
12	Pro-BNP	Pro-brain natriuretic peptide
13	pVASP	Phosphorylated VASP
14	q-RT-PCR	Quantitative real-time reverse transcriptase-polymerase chain reaction
15	RER	Respiratory exchange ratio
16	SCX	Strong Cation Exchange
17	TG	Triglyceride
18	TLC	Thin layer chromatography
19	TMT	Tandem mass tags
20	TnT	Troponin T
21	UCP3	Mitochondrial uncoupling protein 3
22	VASP	Vasodilator-stimulated phosphoprotein
23	VCO₂	CO ₂ production
24	VO₂	Oxygen consumption
25	VLDLR	Very low-density lipoprotein receptor
26	ZDF	Zucker Diabetic Fatty
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1. Introduction

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2 The metabolic syndrome preceding type 2 diabetes is mainly caused by an inefficient
3 coordination of energy utilization by key organs [1]. It is known that alterations in fatty
4 acid (FA) metabolism play a crucial role in inter-organ dyscoordination and insulin
5 resistance (IR) [2]. Further, in metabolic diseases, the heart shows an exacerbated
6 uptake of free FAs (FFAs) [3] and lipoproteins [4] underlying neutral lipid accumulation
7 in cardiomyocytes.
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11 The low-density lipoprotein receptor-related protein 1 (LRP1) is a key receptor that
12 modulates energy utilization by different tissues. LRP1 is both a carrier and a signaling
13 receptor that regulates processes related with lipid metabolism and glucose
14 homeostasis [5,6]. In the hallmark of lipid metabolism, LRP1 is involved in the uptake
15 of unmodified and modified lipoproteins in different tissues causing alterations in the
16 lipid content, generally associated with dyslipemic conditions. LRP1 is involved in the
17 lipid transport from lipoproteins to hepatocytes [5], coronary vascular smooth muscle
18 cells [7,8], cardiomyocytes [9,10] and adipocytes [11]. Specifically, adipocyte LRP1
19 modulates not only lipid transport but also glucose homeostasis through the control of
20 insulin sensitivity and energy expenditure [11]. In the context of glucose homeostasis,
21 LRP1 has a regulatory action on the insulin receptor and GLUT4 activation in different
22 tissues and organs including the brain [12,13]. Interestingly, insulin favors LRP1
23 translocation to the cell membrane in the liver, increasing thus postprandial lipoprotein
24 uptake [14]. Recently, it has been shown that hepatic LRP1 protects against IR and
25 hepatic steatosis [13,15].
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29 The liver plays a central role in the regulation of metabolism, receiving signals from
30 multiple organs such as the pancreatic islets, the central nervous system, adipose
31 tissue and kidneys [16]. It has also been suggested that the heart may also exert
32 control over the metabolism of peripheral organs through the secretion of certain
33 molecules called cardiokines. These cardiokines can affect the metabolic function of
34 various cell types influencing whole-body homeostasis [17–19]. However, it is unknown
35 at this moment whether *lrp1* in cardiomyocytes modulates the production and release
36 of cardiokines potentially involved in whole-body metabolism. The objectives of this
37 study have been to 1) generate a murine experimental model of conditional *Lrp1*
38 deficiency in cardiomyocytes by using the *TnT-iCre* transgenic mice with thoroughly
39 tested suitability to delete genes exclusively in cardiomyocytes [20]; 2) study the impact
40 of cardiomyocyte *Lrp1* deficiency in whole-body metabolism; 3) analyze the differential
41 proteins in the heart of *cm-Lrp1^{-/-}* mice and controls and 4) examine the impact of
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1 differential proteins, in particular of cardiokines, on target-tissue fatty acid uptake and
2 metabolism.

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6 **2. Material and methods**

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8 ***cm-Lrp1^{-/-}* transgenic mice generation and characterization**

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10 We generated an experimental mouse model with conditional and cardiomyocyte-
11 specific *Lrp1* deficiency (TNT-iCre⁺-LRP1^{fl/fl} or *cm-Lrp1^{+/+}*) by treatment with
12 doxycycline [20]. The specific controls for this animal model are TNT-iCre⁻-Lrp1^{fl/fl} or
13 *cm-Lrp1^{-/-}*. The generation of *cm-Lrp1^{-/-}* transgenic mice is described in online Figure
14 S1. To achieve cardiomyocyte-specific LRP1 inactivation, we use a cardiomyocyte-
15 specific Cre deleter and a floxed allele of *Lrp1*. We crossed commercial LRP1^{fl/fl}
16 mice (B6;129S7-Lrp1^{tm2Her}/J; stock: #012604, Jackson Laboratories) carrying loxP sites
17 within the LRP1 gene with TNT-iCre transgenic mice (kindly provided by Prof. Bin
18 Zhou, Albert Einstein College of Medicine, USA) [20], for eight generations (Figure 1A).
19 Transgenic founder mice were genotyped by PCR analysis on tail tip genomic DNA
20 using primers for LRP1, *Cre* and *Nrt* (Figure 1B). Genomic DNA was extracted with a
21 Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA) and
22 PCR analysis was performed with oligonucleotides synthesized by IDT (Integrated
23 DNA Technologies, Inc; Coralville, IA, USA), using an Expand High Fidelity PCR
24 System (Roche Molecular Systems). The oligonucleotides used are specified in Table
25 S1.

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27 Mice were housed in specific pathogen-free facilities with a 12-hour light/12-hour dark
28 cycle. Both groups, ten-week-old males *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}*, were treated daily
29 with doxycycline cyclate (Sigma-Aldrich) (Dox) through their drinking water (1.5
30 mg/mL/kg) *ad libitum* to assess the conditional *Lrp1* inhibition in cardiomyocytes. The
31 control group (*cm-Lrp1^{+/+}*) was also treated with Dox to avoid interference with the
32 treatment. Robertson et al., showed that Dox has a long half-life [21].

33 In the first experimental setting (online Figure S1A), after checking cardiac function,
34 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* ten-week-old mice were fed high-fat cholesterol-containing
35 diet (HFD TD.88137; Harlan Teklad) for six weeks. In the second experimental setting,
36 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* ten-week-old mice were randomized to placebo (PBS) or to
37 NPRA antagonist (A71915, 200 µg/Kg in PBS, volume 100 uL) treatment during the 6
38 weeks on HFD (online Figure S1B). Age-matched littermates were used for all
39 experiments and at least 6 mice per group were analyzed considering $\alpha=0.05$,
40 power=80%, and an effect size of 2 mM glucose and 0.1 µg/L insulin.

1 Blood samples were taken in fasting conditions in the fourth week (150 μ l) and after
2 sacrificing (400 μ L). The serum was frozen at -20°C and used to measure glucose,
3 insulin and lipid levels. The mice were euthanized at week six and samples from liver,
4 skeletal muscle, heart, epididymal fat (eWAT), and brown adipose tissue (BAT), were
5 kept frozen at -80°C for lipid and molecular analysis. A piece of these tissues was also
6 embedded in OCT for immunohistochemistry. All experimental protocols were
7 approved by the Institutional Animal Care and Use Committees at the Research
8 Institute (IR)-Sant Pau, and complied with all guidelines concerning the use of animals
9 in research and teaching as defined by the Guide for the Care and Use of Laboratory
10 Animals (NIH Publication N°.80-23, revised 1996).
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13 **Biochemical analysis**

14 Insulin serum levels were measured using a Mouse Insulin ELISA (Mercodia) following
15 the manufacturer's instructions. The HOMA index, an estimation of insulin resistance,
16 was calculated as: [fasting serum insulin (ng/ml) x fasting serum glucose (mM)]/22.5.
17 Serum lipids and lipoproteins, including cholesterol, triglyceride (TG) (corrected from
18 free glycerol), phospholipids, FFAs, high density lipoprotein (HDL) cholesterol and
19 phospholipids, were enzymatically determined using commercial kits adapted to a
20 COBAS 6000 autoanalyzer (Roche Diagnostics) [22].
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23 **Analysis of ventricular function**

24 Transthoracic echocardiography was conducted under light sedation (1% isoflurane in
25 oxygen) three days after starting doxycycline treatment (baseline). A second
26 echocardiography was conducted six weeks later, at the end of the experimental
27 procedure [23]. Standard functional parameters were measured, including left ventricle
28 (LV) internal diameter diastole (LVIDd) and LV internal diameter systole (LVIDs), LV
29 end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV ejection fraction
30 (LVEF), LV fractional shortening (LVFS), LV anterior wall at end cardiac diastole
31 (LVAWd), LV posterior wall at end cardiac diastole (LVPWd), and heart rate.
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34 **Glucose tolerance test (GTT)**

35 The glucose tolerance test (GTT) was performed at week six of the dietetic intervention
36 in fasting conditions. Basal blood glucose levels were measured from a tail nick
37 through ACCU-CHEK® Aviva glucometer (Roche Molecular Systems). The mice were
38 then intraperitoneally injected with glucose (1.3mg/g BW). Blood glucose was
39 measured at 15, 30, 60, 120 and 180 min after glucose injection. The area under the
40 curve was calculated using the trapezoidal rule.
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curve (AUC) of the response curve was then calculated using the software Prism 4.0 [24].

Distribution of intragastrically-administered [³H]-TG

Mice were given an oral fat gavage (OFG) consisting of 20 μ Ci [³H]-labeled triolein (glycerol tri[9, 10(n)-3 H]oleate, 21.0Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) in 150 μ L of olive oil [24]. Mice were bled by cardiac puncture at 1.5 hours. Serum and target tissues (liver, eWAT, heart, leg muscle and BAT) were collected after perfusion with NaCl solution 0.9%. Radiolabeled serum and tissue TG were separated from the FA using methanol:chloroform:heptane 1.4:1.25:1 (v:v:v) and 0.1 mol/L H₃BO₃-KCO₃ at pH 10.5. The radioactivity in the TG and FA fraction, in total serum and target tissues was determined by scintillation counting.

Cardiomyocyte isolation

Cardiomyocytes were obtained from the heart of our *in vivo* animal model by the Langendorff method. First, the heart was surgically removed and washed with a Ca²⁺ free Tyrode solution at 4°C and oxygenated. The aorta was suspended in the Langendorff apparatus and the heart was perfused via the aorta with Ca²⁺ free Tyrode solution at 37°C to clean blood remnants. The Ca²⁺ free Tyrode solution was then carefully removed, and the enzyme solution composed of Ca²⁺ free Tyrode solution with bovine serum albumin, collagenases and proteinases was added. Two reperfusion cycles (3 ml/min during 6 min) were performed to add the stop solution (Ca²⁺ free Tyrode solution without collagenases and proteinases). Once the perfusion was finished, the heart was removed from the Langendorff system and treated with an enzymatic solution for 5 min at 37°C under shaking. The pieces of tissue were extracted with tweezers and, with shaking, submerged into stop solution to continue disaggregation. Tissue pieces were then removed and the solution containing the cardiomyocytes was centrifuged for 5 min at 450 rpm. The cell pellet was resuspended in stop solution or TripureTM Isolation Reagent®, depending on the further methodology used.

Tissue homogenization

Frozen tissues (25 mg) (heart, liver, skeletal muscle, eWAT and BAT) were pulverized in liquid nitrogen using a mortar and a pestle. Samples were then homogenized in TriPureTM isolation reagent (Roche Molecular Systems) for total RNA and protein extraction according to the manufacturer's instructions. Total RNA from skeletal muscle

1 was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's
2 instructions after the previous addition of Proteinase K. For RNA extraction from
3 eWAT, the tissue was processed with the kit RNeasy Mini Kit (Qiagen). DNA was
4 digested with DNase I (Invitrogen). Extracted RNA was eluted in 25 µL of nuclease-free
5 water. The yield and quality of RNA was tested by agarose electrophoresis and
6 spectrophotometry. Isolated RNA was stored at –80°C until use.
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12 **LRP1-deficient cardiomyocyte cell culture**

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11 Murine LRP1-deficient HL-1 cells were generated and kept in culture as previously
12 described [9,10,25,26].
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16 **Hepatocyte (Hepa 1-6) cell culture**
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18 Hepa 1-6 cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-
19 glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin. Quiescent cells were
20 used for two different experimental approaches. First, quiescent Hepa 1-6 were
21 exposed to supernatants from LRP1- and LRP1- HL-1 cells. Second, quiescent Hepa
22 1-6 were exposed to increasing dose of ANP (0 to 10 nM).
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26 **Gene expression analyses by RT-PCR**
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28 Total RNA (1 µg) was used for cDNA synthesis according to the protocol provided with
29 the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was
30 stored at –20C until its use. Gene expression analyses of *Lrp1* (Mm00464608_m1),
31 *Vldlr* (Rn01498166_m1), *Ldlr* (Mm01151339_m1), *Cd36* (Mm01135198-m1), *Nppa*
32 (Mm01255747_g1), *Nppb* (Mm01255770_g1), *Cpt1* (Mm00550438), *Fasn*
33 (Mm00662319_m1), *Acaca* (Mm01304257_m1), *Acsl3* (Mm01255804_m1), *Cav1*
34 (Mm00483057_m1), *Slc27a2* (Mm00449517_m1) and *Slc27a4* (Mm01327405_m1)
35 were performed by quantitative real-time reverse transcriptase-polymerase chain
36 reaction (q-RT-PCR) in the Applied Biosystems 7300 Real Time PCR System (Applied
37 Biosystems; Foster City, CA, USA). 18srRNA (4319413E) was used as a
38 housekeeping gene. The mRNA expression levels were measured in triplicate. The
39 threshold cycle (Ct) values were normalized to the housekeeping gene.
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43 **Proteomic studies**
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45 Proteomic studies were performed as previously described with small modifications
46 [27,28]. The mass spectrometry proteomics data have been deposited to the
47 ProteomeXchange Consortium via the PRIDE partner repository with the dataset
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1 identifier PXD011564. The protein was digested with Sequencing Grade Modified
2 Trypsin (Promega, Madison, WI, USA) using the FASP (Filter Aided Sample
3 Preparation) digestion protocol as described [27]. A detailed description of the
4 experimental procedures has been included in supplemental material.
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9 **Immunoprecipitation Assays**

10 Dynabeads protein G was bound to the polyclonal anti-corin antibody (abcam
11 ab125254) for 1 hour at room temperature. The protein extract was incubated with the
12 Dynabeads Protein G-anti-corin complex overnight at 4°C. After incubation, the protein
13 immune-complex beads were washed 3 times with the wash buffer and eluted with 50
14 mM glycine. Samples were separated by SDS-PAGE and transferred blots were
15 incubated with Ab against Serpin 1 (biorbyt orb319062).
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19 **Corin catalytic activity assay.**

20 Protease corin activity was measured as previously described [29]. Corin activity was
21 presented as V_{max} .
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24 **Immunoassays**

25 ANP (ab108797, Abcam), GDF-15 (MGD150, R&D Systems) and
26 adrenalin/epinephrine (abx257158, Abbexa) plasma levels were measured by
27 commercially available-enzyme-linked immunosorbent assays (ELISA), according to
28 the manufacturer's recommendations.
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31 **Western blotting analysis**

32 Blots were incubated with antibodies against mouse LRP1 (β -chain, clone 5A6 RDI-
33 PRO61066), AMPK (Cell Signaling Technology, Inc, # 2532), pAMPK (Cell Signaling
34 Technology, Inc, Thr172, 40H9, # 2535), pVASP (Santa Cruz Biotechnology, Inc, sc-
35 101439), BNP (Santa Cruz Biotechnology, Inc, sc-67455), ANP (Everest Biotech,
36 EB11166), Serpin A1 (Biorbyt, orb319062), pACC (Cell Signaling Technology,
37 1673661S, total ACC (Cell Signaling Techonology, 1673662S), pAkt (Cell Signaling
38 Technology, #4060), total Akt (Cell Signaling Technology, #4685), UCP3 (Abcam,
39 ab10985) and OXPHOS mitochondrial complexes (Abcam, ab110413). CPT1 from
40 mitochondrial-enriched fractions in liver was determined [30] using the antibodies
41 described by Herrero L *et al* [31] and registered in antibodyregistry.org (Dolors
42 Serra/Universitat de Barcelona Cat# CPT1A, RRID:AB_2636894). Protein extracts (10
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μg) were loaded, resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes (BioRad). Signal detection was carried out with the ECL immunoblotting detection system (GE Healthcare) and the results were quantitatively analyzed using Chemidoc (BioRad). Equal protein loading in each lane was verified by incubating blots with monoclonal antibodies against troponin T (TnT) (Thermo Scientific #MS-295-P) or β-tubulin (Cell Signaling Technology, Inc, #2146).

Measurement of cyclic GMP levels in tissues.

The cyclic guanosine monophosphate (cGMP) levels in heart, liver and skeletal muscle were measured using an ELISA kit according to the manufacturer's instructions for non-acetylated methodology (ADI-900-014, Enzo Life Sciences).

Confocal microscopy

Mouse heart cryosections were subjected to target retrieval (10 mM Tris-HCl, pH=6) and permeabilized with Triton X-100 (0.5%). Isolated cardiomyocytes were fixed with paraformaldehyde and preserved in PBS with sodium acid 0.2%. Cells were permeabilized with glycine (0.1M) and Triton X-100 (0.2%). Samples were blocked with PBS, Tween-20 (0.2%) and BSA (1%) and incubated with primary antibody LRP1 (2 μg/mL Abcam). Secondary antibodies conjugated with Alexa488 and Cy3 (1 ug/mL) (Jackson ImmunoResearch) were used for detection. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Results were analyzed with an Axio-Observer Z1 (Zeiss) laser confocal microscope.

Histological staining

Tissue samples were frozen and sectioned (5 μm). Sections were stained with Hematoxilin/Eosin, Herxheimer, Oil red O and Sirius red stains. Herxheimer and oil red O stainings were used for the detection of lipids. A polarized light microscope was used to measure collagen types I and III on randomly selected Sirius red-stained sections.

Lipid extraction and semi-quantitative analysis of cholestryly ester, free cholesterol, TG and FFA content in target tissues

Frozen pulverized tissue (10 mg) were homogenized in NaOH 0.1 M. Lipids were extracted and cholestryly esters (CE), free cholesterol (FC) and TG content was analyzed by thin layer chromatography [9,10]. The FFA level was determined enzymatically using commercial kits adapted to a COBAS 6000 autoanalyzer.

Determination of fatty acid β -oxidation (FAO) activity in target tissues

Aliquots from heart and liver were homogenized in a buffer composed of 150 mM NaCl, 1 mM dithiothreitol, 30 mM EDTA and 50 mM KH₂PO₄. Tissue fatty acid oxidation (FAO) was determined with 30 μ g of post nuclear supernatant by determining the conversion of palmitoyl CoA-1-[¹⁴C] into acetyl-CoA [32].

Indirect Calorimetry System

Measurements of oxygen consumption (VO₂) and CO₂ production (VCO₂) were performed using a comprehensive lab animal monitoring system (Oxymax-CLAMS, Columbus Instruments). Mice were acclimated in metabolic chambers for 1 day before the start of the recordings. Animals were continuously recorded for 2 days with measurements of their locomotor activity (in the xy and z axes) and gas exchange (O₂ and CO₂) taken every 20 min. Energy expenditure was calculated according to manufacturer's guidelines (Columbus Instruments). The respiratory quotient was estimated by calculating the ratio of CO₂ production to O₂ consumption.

Statistical analysis

Results are expressed as mean \pm SEM. Differences between study groups were analyzed using one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey-*b* test, Student's *t*-test for independent samples and Student's *t*-test for paired samples. The statistical software R (www.r-project.org) was used for all statistical analyses. Differences were considered statistically significant when *P* < 0.050.

3. Results**3.1. Cardiomyocyte *Lrp1* Deficiency Prevents Diet-Induced Overweight and Glucose Intolerance By Facilitating Increased Energy Expenditure**

Cardiomyocyte *Lrp1* deficient (*cm-Lrp1*^{-/-}) mice were generated as explained in section 2.1 using the *TnT-iCre* transgenic mice provided by Zhou and Bu [20]. These authors showed that the transgenic method they used to generate *TnT-iCre* transgenic mice guarantees the deletion of genes exclusively in cardiomyocytes through all the stages of heart development. Here, we investigated the effects of *Lrp1* specific deletion in cardiomyocytes by crossing *TnT-iCre* transgenic mice with *Lrp1*^{fl/fl} that generated *cm-Lrp1*^{-/-} mice. A schematic representation of *cm-Lrp1*^{-/-} generation and genotyping is shown in Figure 1A,B. *cm-Lrp1*^{-/-} mice fed either chow or HFD diet showed decreased

1 *Lrp1* levels in isolated cardiomyocytes and in the heart (Figure 1C-E). However, not
2 differences were found in other lipoprotein receptors including *vldlr* or *ldlr* in the heart of
3 *cm-Lrp1*^{-/-} and control mice (*online* Figure S2A). *Lrp1* levels in liver, skeletal muscle and
4 eWAT did not show differences between *cm-Lrp1*^{-/-} and control mice (*online* Figure
5 S2B-D).

6
7 First, we evaluated the effects of cardiomyocyte-specific Cre deleter on *Lrp1*
8 expression in chow-fed animals treated with doxycycline. These mice usually exhibit
9 moderate increases in weight and IR when fed a chow diet, but sharp increases when
10 fed a Western-type diet [22]. Surprisingly, the body weight of *cm-Lrp1*^{-/-} mice was lower
11 than that of the controls when the animals were fed a chow diet for a period of 6 weeks
12 and this was concomitant with lower insulin levels and improved glucose tolerance as
13 well as lower hepatic fat accumulation and eWAT weight (*online* Figure S3A-F). Thus,
14 we used western diet-fed *cm-Lrp1*^{-/-} mice to evaluate a putative role of cardiomyocyte
15 *Lrp1* deficiency on metabolic phenotype and the potential mechanisms involved. *Lrp1*
16 deficiency did not cause significant alterations in functional or structural properties of
17 the heart (*online* Figure S4 and S5) or in serum lipid profile (*online* Table S2).

18
19 The body weight of *cm-Lrp1*^{-/-} mice was lower than that of the controls at all tested
20 times (Figure 2A) while the average weekly food intake was similar between both
21 groups (*online* Figure S6). This suggests that the reduction in body weight gain was not
22 due to differences of weekly food consumption. The weight-reducing effect of *Lrp1*
23 deficiency was associated to decreased eWAT weight and eWAT/BAT adipocyte size
24 (Figure 2A-C). While TG/FA content was reduced in the liver of *cm-Lrp1*^{-/-} mice (Figure
25 2D), no differences were found in the heart and skeletal muscle of both groups (*online*
26 Figure S7). To know whether these phenotypic changes elicit a favorable metabolic
27 profile, we conducted a TTG test. Compared with controls, *cm-Lrp1*^{-/-} mice had lower
28 glucose intolerance and lower AUC values (Figure 2E). Glucose, insulin and IR index
29 (HOMA-IR) were reduced in *cm-Lrp1*^{-/-} compared to control mice (Table 1).

30
31 To determine whether cardiomyocyte *Lrp1* deficiency reduces body weight and
32 adiposity by regulating energy expenditure, we performed Comprehensive Lab Animal
33 Monitoring System (CLAMS) experiments. These experiments showed that the
34 ambulation activity was higher during the dark period than during the light period.
35 There were, however, no differences between *cm-Lrp1*^{-/-} and control mice (*online*
36 Figure S8), indicating that the protection against weight gain of *cm-Lrp1*^{-/-} mice was not
37 caused by increased physical activity. *cm-Lrp1*^{-/-} mice showed higher lipid oxidation

1 during the light phase (Figure 3A) and higher glucose oxidation during the dark phase
2 (Figure 3B). *cm-Lrp1^{-/-}* mice showed higher VO₂ and energy expenditure than controls
3 during both phases (Figure 3C & D).

4

5

6 **3.2. Hearts with *Lrp1* Deficiency Show Enhanced Corin Activity That Favors**
7 **Higher ANP Release to the Plasma**

8 To identify cardiac proteins potentially involved in the favorable metabolic phenotype of
9 *cm-Lrp1^{-/-}* mice, we performed proteomic analysis of hearts from both *cm-Lrp1^{-/-}* and
10 *cm-Lrp1^{+/+}* mice groups. We processed three biological replicates from each group.
11 Each replicate was a pool from three animals. We identified 24,358 spectra
12 corresponding to 6,334 non-redundant peptides through a database search (1% FDR)
13 (Data is available via the ProteomeXchange with the identifier PXD011564). For
14 quantitative analysis, only peptides identified as unique (i.e. peptides sequences
15 belonging to one single protein in the database) were considered. Overall, a total of
16 1,213 proteins were quantified from 5,735 non-redundant unique peptides. Quantified
17 proteins were compared between groups and we found a total of 34 differential
18 expressed proteins. These 34 proteins included 11 which have the potential to be
19 secreted (Table 2). Among the differential secreted proteins, we found atrial natriuretic
20 peptide (ANP) (ratio=0.58; p=2.1E-04) and serine protease inhibitors including
21 serpin1a (ratio=0.71; p=2.0E-12), serpin 1b (ratio=0.54; p=6.0E-05), serpin 1d
22 (ratio=0.33; p=1.7-E-03) and serpin 3K (ratio=0.71; p=4.0E-09). To validate the
23 Proteomics results, Western blot and immunoprecipitation experiments were
24 performed.

25 As shown in Figure 4A, ANP is generated from the precursor form proANP through the
26 activation of corin, a convertase that facilitates the release of ANP (28 aa) from the C-
27 terminal end of proANP [33]. Western blot analysis performed with Abs that bind to the
28 sequence RIGAQSGLGCNSFR at the C-terminal end of precursor form showed that
29 proANP (14 kDa) levels were strongly decreased in *cm-Lrp1^{-/-}* compared to control
30 mice. This same test also showed preproANP (17 kDa) to be similar among the groups
31 (Figure 4B). These results confirm proteomic differences (decreased levels of proANP
32 in hearts from *cm-Lrp1^{-/-}* mice) and suggest that there is higher release of ANP from
33 LRP1- hearts.

34

35

36 Immunoprecipitation studies evidenced that serpins form complexes with corin. The
37 number of SerpinA1/Corin complexes were higher in the heart of *cm-Lrp1^{-/-}* mice
38 compared to that of controls (Figure 4C). The molecular weight of these complexes in
39

1 the heart of our murine model was 45-50 KDa and 30-35 KDa, which lines up with what
2 was previously described of cultured cardiomyocytes [34]. Gladysheva IP *et al* reported
3 that serpin1 binding to corin creates steric impediments to the union of effective
4 inhibitors and allows prolonged corin activation [34].
5
6

7 Fluorogenic assays performed in this study revealed that protease enzymatic activities
8 were higher in the cardiac membranes of *Lrp1*^{-/-} mice with or without hirudin (Figure
9 4D). Hirudin, a highly specific thrombin inhibitor, was added to the assay to exclude
10 clotting enzymes in the membrane fraction. It did not show a significant effect on
11 protease activity, suggesting that corin could be one of the main proteases contributing
12 to the higher protease activity in cardiac membranes of *Lrp1*^{-/-} mice.
13
14

15 Circulating ANP levels were higher (about 4-fold) in the plasma of *cm-Lrp1*^{-/-} than in
16 that of *cm-Lrp1*^{+/+} mice (Figure 4E). Our results suggest that cardiac *Lrp1* deficiency
17 facilitates corin activation and ANP release. There were no differences in cardiac *Nppa*
18 between groups, whereas a modest downregulation in *Nppb* mRNA expression was
19 found in *cm-Lrp1*^{-/-} mice (*online* Figure S9). No differences were found in the circulating
20 levels of other metabolic factors including adrenalin/epinephrine and GFD15 (Figure
21 4F,G).
22
23

33 **3.3. The ANP-NPR-A Signaling Activation Is Linked To AMPK Phosphorylation 34 and Increased FA Oxidation in the Liver of *cm-Lrp1*^{-/-} Mice**

35 To ascertain whether increased circulating ANP levels were associated with the
36 activation of NPR-A signaling in peripheral tissues, we determined the levels of crucial
37 mediators such as cGMP and pVASP in the liver, skeletal muscle and heart of *cm-*
38 *Lrp1*^{+/+} and *cm-Lrp1*^{-/-} mice whether treated with the NPR-A antagonist A71915 or not
39 (*online* Figure S1B). cGMP and VASP were increased in the liver (Figure 5A,B) and
40 skeletal muscle (*online* Figure S10A,B) but not the heart (*online* Figure S11A,B) of
41 untreated *cm-Lrp1*^{-/-} mice. The treatment of *cm-Lrp1*^{-/-} mice with A71915 completely
42 blocked the increase of cGMP and pVASP levels in liver and partially blocked it in
43 skeletal muscle. We found that cGMP/pVASP signaling was linked to increased AMPK
44 phosphorylation in the liver. Increased levels of hepatic pAMPK, like those of cGMP
45 and pVASP, were abolished by the NPR-A antagonist (Figure 5A,B). There were no
46 differences in pAMPK levels in the skeletal muscle (*online* Figure S10B) or the heart
47 (*online* Figure S11B) between groups. To know whether AMPK phosphorylation was
48 linked to AMPK activity, we measured the phosphorylation of ACC, the downstream
49 target of pAMPK. ACC mRNA expression levels in the liver of mice fed HFD were
50
51

extremely low (reduced by approx. 90% in HFD fed mice). Despite this, we detected an increased ratio pACC/total ACC in the liver of untreated *cm-Lrp1^{-/-}* compared to the control mice (Figure 5A). In addition, the increase in the phosphorylation of ACC was not observed in A71915-treated mice, supporting the activation of AMPK by ANP in the liver of KO mice. To explore the potential impact of pAMPK activation on hepatic FA synthesis, we explored several genes involved in FA synthesis in the liver. We found that *Acs3* mRNA expression was significantly lower in the liver of KO mice compared to the controls (*online* Figure S12). There were no differences in the mRNA expression levels of *Fasn* or *Slc27a2* between groups.

To know the impact of pAMPK on FAO, we measured CPT1, a key mitochondrial enzyme responsible for FAO; the main components of the oxidative phosphorylation system (OXPHOS), responsible for mitochondrial respiration; and UCP3, an indicator of mitochondrial response to enhanced intracellular FA. Oxidative phosphorylation involves a flow of electrons through the electron transport chain (ETC), a series of proteins and electron carriers within the mitochondrial membrane. We used one OXPHOS cocktail antibody that contains 5 mouse Abs and detects one subunit of each (five) mitochondrial complexes of the ETC. The main subunits detected in whole hepatic and skeletal muscle tissue extracts of our murine model were CV-ATP5A (55 kDa), CIII-UQCRC2 (48 kDa) and CII-SDHB (30 kDa). The protein levels of CPT1, CII, and CV subunits of ETC were significantly higher in the liver (Figure 5C) of untreated KO mice. The increase in these mitochondrial proteins was abolished in *cm-Lrp1^{-/-}* mice treated with A71915, indicating that NPRA-signaling is involved in the increase of OXPHOS protein subunits in mice. UCP-3, an indicator of FA supply to mitochondria, was also induced in the liver (Figure 5C) but not in the skeletal muscle (*online* Figure S10C) of KO mice. These results suggest that, despite the upregulatory effect of ANP signaling on OXPHOS subunits of the skeletal muscle, ANP signaling is not linked to FA oxidation in the skeletal muscle.

According to CPT1 upregulation, the hepatic FAO activity was higher in untreated KO mice than in control mice, but this increase was not observed in KO mice treated with A71915 (Figure 5D). In addition, KO mice showed reduced hepatic TG accumulation in the untreated mice but not in the A71915-treated mice (Figure 5E).

To explore whether these favorable changes may influence insulin signaling activation, we measured the activation state of Akt in different tissues. We found an increased ratio of pAkt/total Akt exclusively in the liver of KO mice (*online* Figure S13).

3.4. Supernatants from LRP1 deficient HL-1 Cells Increased pAMPK and CPT1 Levels in Cultured Hepatocytes

LRP1-deficient HL-1 cells (LRP1-) were generated in our group and previously used to test the impact of the cardiomyocyte LRP1 deficiency in several processes [9,10,25,26]. LRP1- cells show almost undetectable levels of LRP1 but similar morphologic characteristics to the control cells (Figure 6A,B). Supernatants from LRP1- cells contain higher ANP levels than those from control cells (Figure 6C), and, when added to hepatocytes, caused induced AMPK phosphorylation and increased CPT1 protein levels in cultured Hepa 1-6 cells (Figure 6D,E). In parallel experiments, we also showed that ANP from 0 to 10 nM caused an increase in pAMPK/AMPK ratio and CPT1 protein levels in a dose-response manner without altering cell morphology (*online* Figure S14A-C).

3.5. Activated ANP-NPR-A Signaling Is Linked to Increased FA Uptake by the Liver

AMPK phosphorylation has been previously reported to activate FA uptake in oxidative tissues [35,36]. Oral fat gavage (OFG) experiments showed an increase in [³H]-TG and [³H]-FFA uptake by the liver of *cm-Lrp1^{-/-}* mice that was blocked by the NPR-A antagonist A71915 (Figure 7A). Real-time PCR experiments showed an increased expression of hepatic *Vldlr* and *Cd36* mRNA in *cm-Lrp1^{-/-}* mice (Figure 7C) that were also efficiently blocked by the NPR-A antagonist. Unlike the liver, there were no differences between KO and control mice in [³H]-TG and [³H]-FFA uptake by the skeletal muscle, although a significant increase in [³H]-TG and [³H]-FFA uptake was detected in groups treated with A71915 (online Figure S15). In contrast to liver, there was a reduced uptake of [³H]-TG and [³H]-FFA by eWAT of *cm-Lrp1^{-/-}* mice (Figure 7B). Reduced levels of *Vldlr* and *Cd36* mRNA expression was detected in eWAT of KO versus control mice (Figure 7D). As shown in Figure 7E,F, A71915 equaled eWAT weight and eWAT adipocyte size between *cm-Lrp1^{-/-}* and *cm-Lrp1^{+/+}* mice.

CLAMS experiments showed that treatment of *cm-Lrp1^{-/-}* mice with the NPR-A antagonist counteracted increased lipid oxidation during the light phase (Figure 8A) and increased glucose oxidation during the dark phase (Figure 8B). There were no differences in oxygen consumption (Figure 8C) and energy expenditure (Figure 8D) between *cm-Lrp1^{-/-}* and control mice treated with the NPR-A antagonists.

4. Discussion

1
2 The main innovative findings of this study are that LRP1 receptor levels in
3 cardiomyocytes regulate circulating levels of atrial natriuretic peptide and that through
4 this mechanism control NPRA-dependent fatty acid metabolism in the liver and,
5 consequently, the whole-body metabolism.
6
7

8
9
10 **LRP1 Controls the Systemic Levels of Atrial Natriuretic Peptide through**
11 **Modulation of Corin Activity in Cardiomyocytes**

12 Here, we show that isolated cardiomyocytes from *cm-Lrp1^{-/-}* mice are deficient in the
13 receptor *Lrp1*. Previous studies have shown that LRP1 plays a crucial role in the
14 degradation of serpin:protease complexes [37,38]. Results from the
15 immunoprecipitation assays performed in this study indicated that corin, the main
16 protease responsible for cardiac release of atrial natriuretic peptide [33], was mostly
17 complexed with serpins in the heart of *cm-Lrp1^{-/-}* mice, as it was previously shown in
18 cultured cardiomyocytes [34]. In agreement with the capacity of LRP1 to modulate
19 protease levels, we found that *Lrp1* deficiency in the cardiomyocyte, the most abundant
20 and main ANP producing cell in the heart [39,40], directly impacts corin activity, cardiac
21 ANP release and ANP circulating levels. A more direct proof of the capacity of LRP1 to
22 modulate ANP release has been obtained from cell culture experiments performed with
23 an LRP1-deficient HL-1 cell line (LRP1- HL-1 cells) previously generated in our group
24 [9,10,25,26]. Our results evidenced the presence of higher ANP levels in the
25 supernatants from LRP1- HL-1 cells compared to control cells.
26
27

28 The protease corin is also involved in the cleavage of pro-brain natriuretic peptide
29 (BNP) to BNP [41]. Despite this, we did not observe alterations in cardiac or plasma
30 BNP levels in *cm-Lrp1^{-/-}* mice compared to the controls. The specific effect of the
31 receptor deficiency on ANP release could be explained by the predominance of ANP
32 (compared to the marginal presence of BNP) in hearts without ventricular dysfunction
33 [42].
34
35

36 Several groups including ours, have determined that the modulation of *Lrp1* receptor
37 causes alterations of key signaling pathways in the heart. Our group reported that
38 cardiac *Lrp1* overexpression contributes to enhanced cholesterol and triglyceride
39 supply to the heart and is associated with serious calcium-handling alterations [25]. In
40 the context of the myocardial infarction, LRP1 signaling activation has been reported to
41 induce a cardioprotective signal, decreases infarct size, and preserves cardiac systolic
42 function in young adult mice without comorbidities [43]. Elevated levels of *Lrp1* ligands,
43 such as VLDL, can act as antagonists of *Lrp1* signaling [44]. Results from the present
44

1 study show that cardiomyocyte *Lrp1* deficiency did not affect in cardiac function. This
2 could be related to the absence of pathological stressors such as atherogenic
3 dyslipemia or hypoxia. Further studies are required to know the optimal *Lrp1* threshold
4 values in the presence of certain pathological ligands
5
6

7
8 **ANP Activates NPR-A Signaling That Is Linked to AMPK Phosphorylation and**
9 **Increased FA Oxidation/FA Uptake in the Liver of *cm-Lrp1*^{-/-} Mice.**
10

11 The systemic effects of ANP are well described, contributing to water-salt balance
12 maintenance and blood pressure regulation through diuretic, natriuretic and
13 vasodilatory effects. In addition, ANP displays important pleiotropic effects in the heart,
14 acting as a main regulator of cardiovascular homeostasis in an autocrine and paracrine
15 manner [45]. Besides the hemodynamic effects, ANP also is involved in lipolytic
16 processes in white adipose tissue in primates [46]. Recently, it has been described as
17 a potent lipolytic agent that exerts a great impact on the metabolism of patients with
18 heart failure [47]. Interestingly, ANP induces anti-inflammatory effects in adipose tissue
19 [48].
20
21

22 ANP has also been reported to promote oxidative metabolism in human skeletal
23 muscle. In particular, ANP induces PGC-1 α and mitochondrial OXPHOS gene
24 expression in a cyclic GMP-dependent manner in human myotubes [49]. Results from
25 the present study show that ANP increased NPR-A/cGMP/pVASP in the skeletal
26 muscle of *cm-Lrp1*^{-/-} mice. In our murine model, this upregulatory effect of ANP
27 signaling was linked to mitochondrial OXPHOS upregulation, affecting CV-ATP5A and
28 CII-SDHB subunit overexpression. However, there were no differences in the levels of
29 phosphorylated AMPK, the TG/FA uptake, the levels of CPT1 and UCP3, and TG
30 accumulation in the skeletal muscle of *cm-Lrp1*^{-/-} and control mice. These results
31 indicate that FA uptake and FA oxidation were not significantly modulated by NPR-
32 A/cGMP/pVASP signaling in the skeletal muscle of *cm-Lrp1*^{-/-} mice.
33
34

35 In contrast, we found that NPR-A/cGMP/pVASP signaling was linked to AMPK
36 phosphorylation in the liver of *cm-Lrp1*^{-/-} mice. Our cell culture experiments evidenced
37 that hepatocytes exposed to supernatants from LRP1- HL-1 cells or to ANP directly
38 showed increased levels of phosphorylated AMPK. Previous studies have shown that
39 pVASP, an ANP signaling mediator, phosphorylated specifically AMPK in the liver of a
40 mouse model of diabetes [50]. AMPK is considered the master regulator of lipid
41 metabolism and mitochondrial homeostasis [51–54]. One of the crucial functions of
42 AMPK is the regulation of lipid metabolism through the phosphorylation of ACC. Upon
43
44

activation of AMPK, phosphorylation of ACC results in ACC inactivation. Our results indicate that ACC phosphorylation is increased in the liver of KO mice and abolished by the treatment of KO mice with the NPR-A antagonist, indicating that the energy sensing AMPK/ACC pathway can be modulated by ANP in the liver. *Acs3* mRNA expression levels were also decreased in the liver of *cm-Lrp1^{-/-}* compared to control mice. *Acs3* knockdown decreased total acyl-CoA synthetase activity in ob/ob mice and mice fed a high sucrose diet [55]. Together, these results support that ANP-induced AMPK phosphorylation is involved in the deactivation of FA synthesis in the liver of *cm-Lrp1^{-/-}* mice.

The phosphorylation of ACC inhibits the production of malonyl-CoA, a substrate for fatty acid synthase (FAS) and a potent inhibitor of CPT1 [56]. CPT1 is the main mitochondrial enzyme responsible for fatty acid oxidation. Results from the present study showed increased levels of CPT1 in the liver *cm-Lrp1^{-/-}* mice and in hepatocytes exposed to increased ANP levels. We also showed that this increase is dependent of NPR-A signaling. The link between AMPK phosphorylation and CPT1 activation has been previously shown in oxidative tissues of a mice model [36,51]. The upregulation of the mitochondrial OXPHOS subunits support and enhanced mitochondrial respiration in the liver of *cm-Lrp1^{-/-}* mice. Further experiments are required to know the mechanism by which ANP signaling enhances the level of these OXPHOS protein subunits. This could be due to enhanced mitochondrial synthesis or enhanced amount of protein subunits per mitochondria. Together these results support an ANP-enhanced mitochondrial fatty acid oxidation in the liver of *cm-Lrp1^{-/-}* mice.

Finally, UCP3, an indicator of mitochondrial response to intracellular FA, [³H]-FA and [³H]-TG uptake and FA/TG transporters such as Cd36 and VLDLR were upregulated in the liver of KO mice and the increase was abolished by the treatment of KO mice with the NPR-A antagonist. Previous studies have reported that pAMPK positively modulates Cd36 in a mouse model of AMPK activation specifically in the liver [57]. Together, these results evidenced that ANP signaling promoted by cardiomyocyte LRP1 deficiency facilitates the repression of FA synthesis and the activation of FA uptake/FA mitochondrial oxidation in an AMPK-coordinated manner in the liver of *cm-Lrp1^{-/-}* mice.

ANP-Induced Hepatic FAO Confers a Favorable Metabolic Profile to *cm-Lrp1^{-/-}* Mice

1 Here, we show that enhanced FA uptake and oxidation promoted by ANP reduces TG
2 accumulation in the liver of KO mice. These results are in agreement with previous
3 studies showing that moderate increases in *Cpt1a* activity are sufficient to reduce
4 hepatic TG levels in CPT-1a-overexpressing animals [58] and even to modulate
5 diabetes and obesity [30]. Interestingly, reduced hepatic triglyceride content in KO mice
6 occurred in the context of enhanced supply of FA to the liver, and, by counteracting
7 ANP-induced FA uptake by the liver, the NPR-A antagonist caused an increased FA
8 uptake by eWAT. Although, a direct effect of ANP on adipose tissue of *cm-Lrp1*^{-/-}
9 cannot be discarded, data from the present study supports the secondary nature of
10 cardiac *Lrp1* deficiency's benefits such as reducing diet-induced obesity and increasing
11 energy expenditure. The liver is considered the main organ in the homeostasis of lipids
12 and glucose and one of the highest contributors to whole-body lipid oxidation [59].
13 Moreover, previous studies have consistently demonstrated the link between the
14 hepatic FAO and insulin-resistance [45,47,54]. Results from the present study show
15 that phosphorylation of Akt, a crucial step in the insulin signaling pathway, is more
16 active in the liver of *cm-Lrp1*^{-/-} mice compared with controls. In addition, CLAMS
17 experiments showed an ANP-induced glucose oxidation during the dark phase.
18 However, further studies based on the analysis of signaling right after insulin
19 administration are indeed required to obtain conclusions about the role of ANP on the
20 modulation of diet-induced hepatic insulin resistance [60]. Similarly, it would be
21 interesting to determine whether the response to leptin is altered in our model.
22
23

36 **Main Conclusions and Translational Potential of the Work**

37 Our results show that the favorable metabolic profile and increased energy expenditure
38 in *cm-Lrp1*^{-/-} mice mainly depends on ANP signaling involving hepatic AMPK activation
39 (summarized in Figure 9).
40

41 From the clinical point of view, our team has evaluated natriuretic peptide levels in
42 patients with chronic heart failure. Recently, we described that patients with a low
43 capacity to degrade active natriuretic peptides had better outcomes [61,62]. In humans,
44 the main protective metabolic effects of ANP are related to ANP lipolytic effects in
45 adipose tissue [46,63–67]. In fact, high levels of natriuretic peptides in plasma have
46 been associated with weight loss in humans [66,67]. Here, treatment of *cm-Lrp1*^{-/-} mice
47 with the NPR-A antagonist restores energy expenditure to the levels of control mice,
48 suggesting that ANP/NPR-A signaling is involved in the increased energy expenditure
49 and reduced weight of *cm-Lrp1*^{-/-} mice. It will be interesting to test in humans whether
50 ANP-induced hepatic FA uptake/oxidation is an additional mechanism contributing to
51 the impact of high circulating NP levels in weight loss. According to the present study,
52

low levels of *Lrp1* expression in cardiomyocytes would contribute to the maintenance of elevated NP levels in circulation. This could be highly relevant in patients with metabolic disorders.

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Conflicts of interest

The authors have declared no potential conflicts of interest.

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- 28 **Figure Legends**
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- 30 **Figure 1. *cm-Lrp1*^{-/-} Transgenic Mice Generation and Validation**
- 31 (A) Generation schedule of an *in vivo* model with a doxycycline-inducible attenuation of
32 *Lrp1* expression selectively in cardiomyocytes. In *cm-Lrp1*^{-/-} mice with the genotype
33 TnTCre^{+/+} *Lrp1*^{fl/fl}, the activation of Cre recombinase by doxycycline converts the
34 *Lrp1* floxed alleles to KO (-) alleles. (B) Cre and Flox transgens were detected by PCR.
35 A fragment of 500 bp was amplified from mouse tail genomic DNA when the Cre
36 transgene driven by the TnT promoter was present. A fragment of 350 bp was
37 amplified when flox sequences flank *Lrp1* gen. (C) Ten-week-old male mice were
38 treated daily with doxycycline cyclate through their drinking water during the procedure
39 to assess the conditional inhibition of LRP1 levels in cardiomyocytes. After three days
40 of treatment, both *cm-Lrp1*^{+/+} and *cm-Lrp1*^{-/-} mice were randomized and fed a chow diet
41 or high fat diet for six weeks. (D) *Lrp1* mRNA expression and LRP1 protein levels (in
42 green) in isolated cardiomyocytes from hearts of *cm-Lrp1*^{-/-} and control mice (E) *Lrp1*
43 mRNA expression and LRP1 protein levels (LRP1 staining in red, nuclei in blue,
44 Bar=20 μ m) in the heart. Data represent the mean \pm SEM for 9 mice per group. ***
45 p<0.005 versus *cm-Lrp1*^{+/+} mice; ### p<0.005 versus chow diet. Differences between
46 groups were analyzed using one-way analysis of variance (ANOVA) followed by a
47 post-hoc Tukey-b test.
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2 **Figure 2. *Lrp1* Deficiency in Cardiomyocytes Improves Diet-induced Glucose**
3 **Intolerance and Reduces Weight Gain**

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5 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline
6 cyclate in their drinking water. (A) Body weight changes over 6 weeks of HFD diet. (B)
7 Epididymal white adipose tissue (eWAT) tissue mass at necropsy. (C) Representative
8 H&E stained sections from eWAT and brown adipose tissue (BAT) (Bar=100 μ m). (D)
9 Triglyceride and FA content in the liver and representative Herxheimer stained liver
10 sections (Bar =100 μ m). (E) Glucose tolerance test in mice (1.3 mg/g body weight) and
11 Area under the curve (AUC). The data represents the mean \pm SEM. n=9 mice per
12 condition. **p<0.01 or *** p<0.005 versus control mice. Differences between groups
13 were analyzed using Student's t test for independent samples.
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19 **Figure 3. Cardiomyocyte *Lrp1* Deficiency Increases Whole-Body Energy**
20 **Consumption**

21 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline
22 cyclate in their drinking water and then submitted to CLAMS experiments. Oxymax
23 respirometer measurements of lipid oxidation (A), glucose oxidation (B), VO₂ (C) and
24 whole-body oxygen consumption (D) during the light and dark periods in mice over 6
25 weeks on HFD. n=8 mice per condition. *p<0.05, **p<0.01 or ***p<0.005 versus *cm-*
26 *Lrp1^{+/+}* mice. Differences between groups were analyzed using Student's t test for
27 independent samples.
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38 **Figure 4. Cardiomyocyte *Lrp1* Deficiency Favors Higher Corin Activity and ANP**
39 **Release**

40 (A) Schematic representation for the secretion of preproANP-derived peptides
41 modified from Pemberton et al [23]. The AA sequence RIGAQSGLGCNSFR that is
42 recognized by the antibody (Ab) is localized at the extreme C-terminal of the protein.
43 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline
44 cyclate in their drinking water. After sacrifice, one aliquote of frozen heart tissue was
45 used for protein extraction. (B) Representative Western blot analysis showing cardiac
46 preproANP (16 kDa), proANP (13 kDa) and troponin (TnT) (endogenous control)
47 protein bands and bar graphs showing the quantification of preproANP and proANP
48 protein bands normalized to TnT. One aliquot of cardiac membranes (5 mg) was used
49 for immunoprecipitation with anti-corin Abs. (C) Immunoblots performed with anti-
50 SerpinA1 Abs showing two different molecular weight corin/serpinA1 complexes and
51 bar graphs showing the quantification of 45-50 kDa complex and 30-35 kDa complex.
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(D) Other aliquot of cardiac membranes (25 µg) was used for membrane protease catalytic activity measurements (Vmax) in absence or presence of the thrombin inhibitor hirudin (2 µM). (E-G) Circulating levels of ANP, adrenalin/epinephrine and GDF15 measured by ELISA. n=6 mice per condition. Data represent the mean±SEM. **p<0.01 or ***p<0.005 versus *cm-Lrp1^{+/+}* mice. Differences between groups were analyzed using Student's t test for independent samples.

Figure 5. High Plasma ANP Levels in *cm-Lrp1^{-/-}* Mice Induce NPRA-Dependent Signaling Linked to AMPK Phosphorylation and Increased Fatty Acid Oxidation in the Liver

cm-Lrp1^{+/+} and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline cyclate in their drinking water and then treated with either saline or the NPRA antagonist A71915 (200 µg/kg in PBS in a final volume of 100 µl) for the last 4 weeks. (A) cGMP levels were detected by ELISA. Western blot analysis and bar graphs showing the quantification of pVASP, total VASP, pAMPK, total AMPK, pACC and total ACC (B) and OXPHOS, UCP3 and CPT1 (C) in the liver. (D) Rate of hepatic fatty acid oxidation (FAO). (E) Representative Herxheimer stained liver sections (Bar =100 µm) and hepatic TG levels. n=8-10 mice per condition. Data represent the mean±SEM. *p<0.05, ** p<0.01 or *** p<0.005 versus *cm-Lrp1^{+/+}* mice; ## p<0.01 or ### p<0.005 vs PBS. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey-b test.

Figure 6. Hepatocytes Exposed to Supernatants from LRP1-deficient (LRP1-) HL-1 Cardiomyocytes Exhibited Increased Levels of Phosphorylated AMPK and CPT1

LRP1 deficient HL-1 (LRP1-) and control HL-1 cells (LRP1+) were grown in complete Claycomb medium from 48 hours and supernatants were then collected. After determination of their ANP content by ELISA, supernatants were directly added to cultured hepatocytes (Hepa 1-6). Hepa 1-6 were exposed to HL-1 supernatants for 18 hours and then collected in lysis buffer to perform Western blot analysis. (A) Representative optical microscopy image of LRP1+ and LRP1- HL-1 cells. (B) Western blot analysis and bar graphs showing LRP1 protein levels in LRP1- compared to LRP1+ HL-1 cells. (C) ANP levels in LRP1+ and LRP1- supernatants measured by ELISA. (D) Representative optical microscopy image of Hepa 1-6 cells exposed to supernatants from LRP1+ and LRP1- HL-1 cells. (E) Western blot analysis and bar graphs showing pAMPK/total AMPK ratio and CPT1 protein levels of Hepa 1-6. Data

1 represent the mean \pm SEM of three experiments performed in duplicate. ** p<0.01 or ***
 2 p<0.005 versus LRP1+ HL-1 cells or versus Hepa 1-6 cells exposed to supernatants
 3 from control HL-1 cells. Differences between groups were analyzed using Student's t
 4 test for independent samples.
 5

6

7 **Figure 7. TG and FA Uptake Increased in the Liver while Decreased in eWAT of**
 8 ***cm-Lrp1^{-/-}* Mice**

9 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline
 10 cyclate in their drinking water and then treated with either saline or the NPR-A
 11 antagonist A71915 for the last 4 weeks. Mice were then given an oral fat gavage
 12 (OFG) consisting of 20 μ Ci [3 H]-labeled triolein in 150 μ L of olive oil as. Percentage of
 13 [3 H]-triglyceride and [3 H]-free fatty acid uptake by liver (A) and eWAT (B) in the different
 14 groups taking as reference the control group. Real-time PCR of *Vldlr* and *Cd36* mRNA
 15 expression levels in liver (C) and eWAT (D). (E) Representative Herxheimer stained
 16 eWAT sections (Bar =100 μ m). Data represent the mean \pm SEM. *p<0.05, **p<0.01 or
 17 ***p<0.005 versus control mice; ## p<0.01 or ### p<0.005 versus PBS. Differences
 18 between groups were analyzed using one-way analysis of variance (ANOVA) followed
 19 by a post-hoc Tukey-b test.
 20

21

22 **Figure 8. Cardiomyocyte *Lrp1* Deficiency Increases Whole-Body Energy**
 23 **Consumption in an NPR-A Dependent manner**

24 *cm-Lrp1^{+/+}* and *cm-Lrp1^{-/-}* mice (control) mice were treated for 6 weeks with doxycycline
 25 cyclate in their drinking water and then treated with either saline or the NPR-A
 26 antagonist A71915 for the last 4 weeks. Oxymax respirometer measurements of lipid
 27 oxidation (A), glucose oxidation (B) VO_2 (C) and whole-body oxygen consumption (D)
 28 during the light and dark periods. n=8-10 mice per condition. *p<0.05, ** p<0.01 or
 29 ***p<0.005 versus control mice; ## p<0.01 or ### p<0.005 versus PBS. Differences
 30 between groups were analyzed using one-way analysis of variance (ANOVA) followed
 31 by a post-hoc Tukey-b test.

32

33 **Figure 9. Schematic main figure**

34 Cardiomyocyte *Lrp1* deficiency facilitates corin activation and ANP release from the
 35 heart. NPR-A/cGMP/pVASP signaling activates AMPK/ACC pathway that coordinately
 36 inhibits FA synthesis and activates FA uptake and oxidation in the liver of *cm-Lrp1^{-/-}*
 37 mice. The ANP-mediated activation of AMPK seems to underlie the decrease in
 38

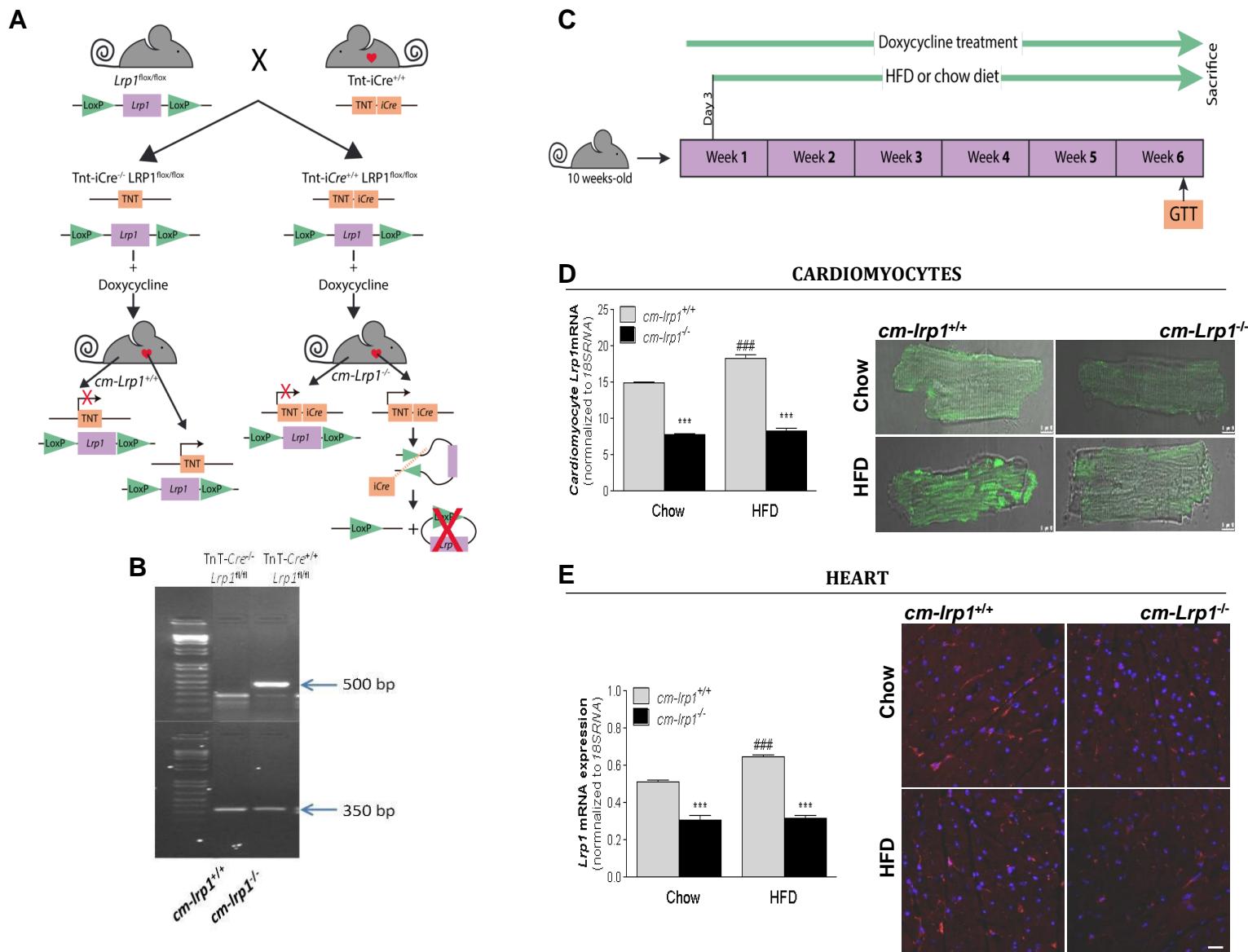
hepatic TG content, the improved glucose tolerance, the limited weight gain and the enhanced whole-body energy consumption of cardiomyocyte-*Lrp1* deficient mice.

Table 1. Comparison of serum glucose levels, serum insulin levels and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) between *cm-Lrp1^{+/+}* and control mice. The data is shown as mean±SEM. N=8 per group. Differences between groups were analyzed using the Student's t test for independent samples.

	<i>cm-Lrp1^{+/+}</i>	<i>cm-Lrp1^{-/-}</i>	P
GLUCOSE (mM)	11.09 ± 0.37	9.49 ± 0.84	0.072
INSULIN (μg/L)	1.23 ± 0.17	0.48 ± 0.04	0.003
HOMA-IR	13.06 ± 1.55	4.59 ± 0.74	0.003

Table 2. Differential cardiac secreted proteins between *cm-Lrp1^{-/-}* and *cm-Lrp1^{+/+}* mice. This table shows mass spectromic proteomic data concerning differential cardiac secreted proteins. Three biological replicates for each condition were processed. Each replicate was a pool of tissue obtained from three different animals. Differences between groups were analyzed using Student's t test for independent samples.

Protein ID	Protein name	Gene name	<i>cm-Lrp1^{-/-}</i> vs <i>cm-Lrp1^{+/+}</i>	
			Ratio	P
E9PV24	Fibrinogen alpha chain	Fga	0.64	1.8E-12
P05125	Natriuretic peptides A (ANP)	Nppa Pnd	0.57	4.8E-04
P07759	Serine protease inhibitor A3K	Serpina3k Mcm2 Spi2	0.63	1.6E-12
P22599	Alpha-1-antitrypsin 1-2	Serpina1b Aat2 Dom2 Spi1-2	0.44	1.2E-05
Q00897	Alpha-1-antitrypsin 1-4	Serpina1d Dom4 Spi1-4	0.30	9.4E-04
Q3TFQ8	Alpha-1,4 glucan phosphorylase	Pygb	0.72	8.1E-16
Q6S9I0	Kininogen	Kng2	0.77	1.1E-03
P01942	Hemoglobin subunit alpha	Hba	0.74	9.6E-05
Q8K0E8	Fibrinogen beta chain	Fgb	0.66	6.6E-14
Q8VCM7	Fibrinogen gamma chain	Fgg	0.65	5.5E-08
Q91X72	Hemopexin	Hpx Hpxn	0.70	5.0E-09



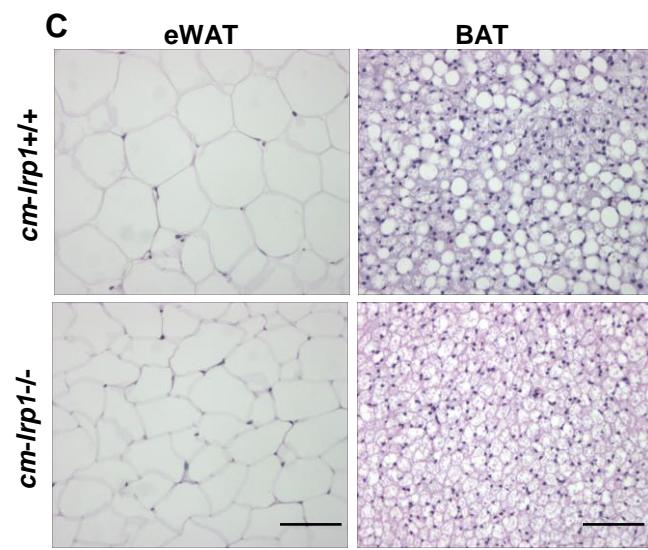
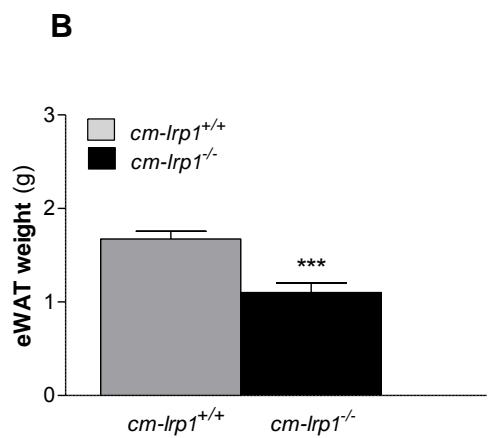
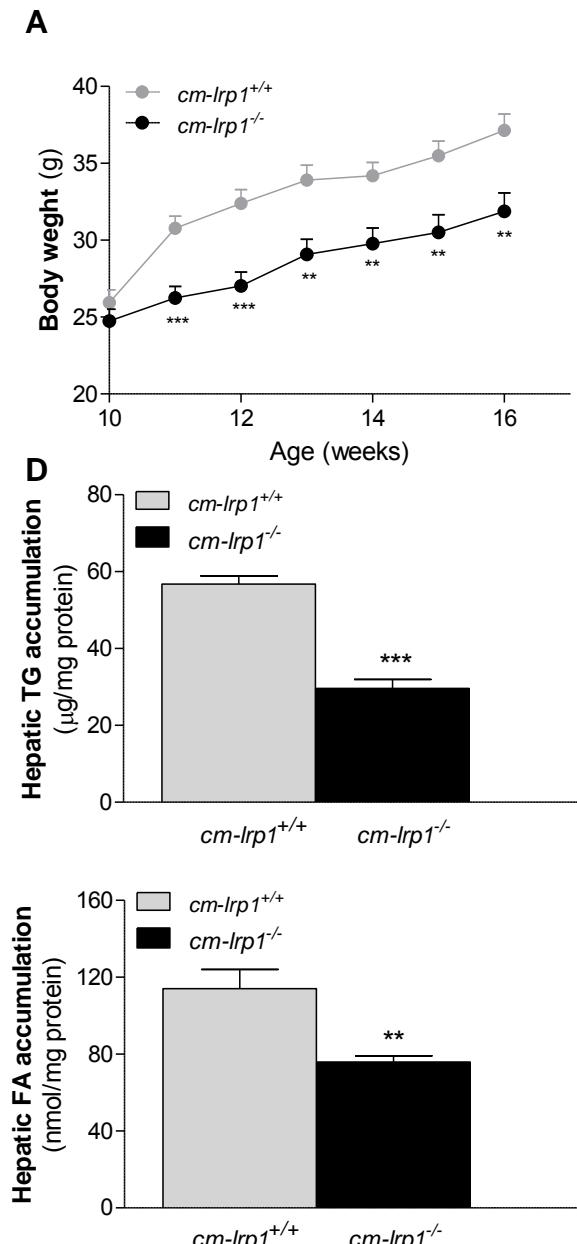
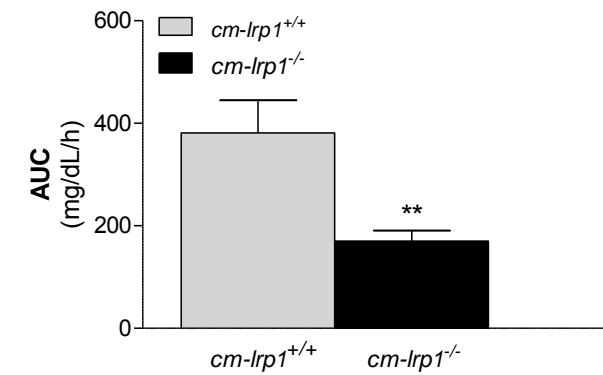
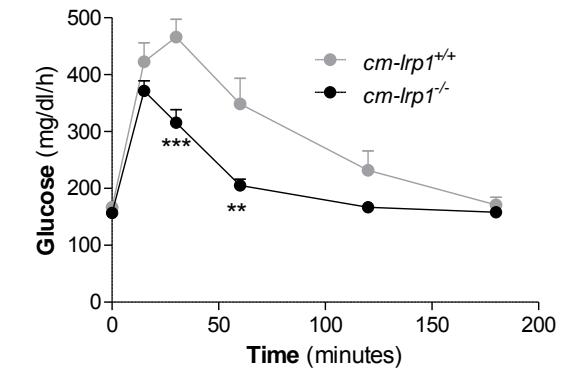
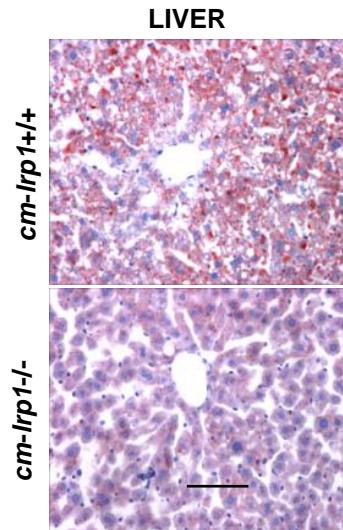
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Figure 3

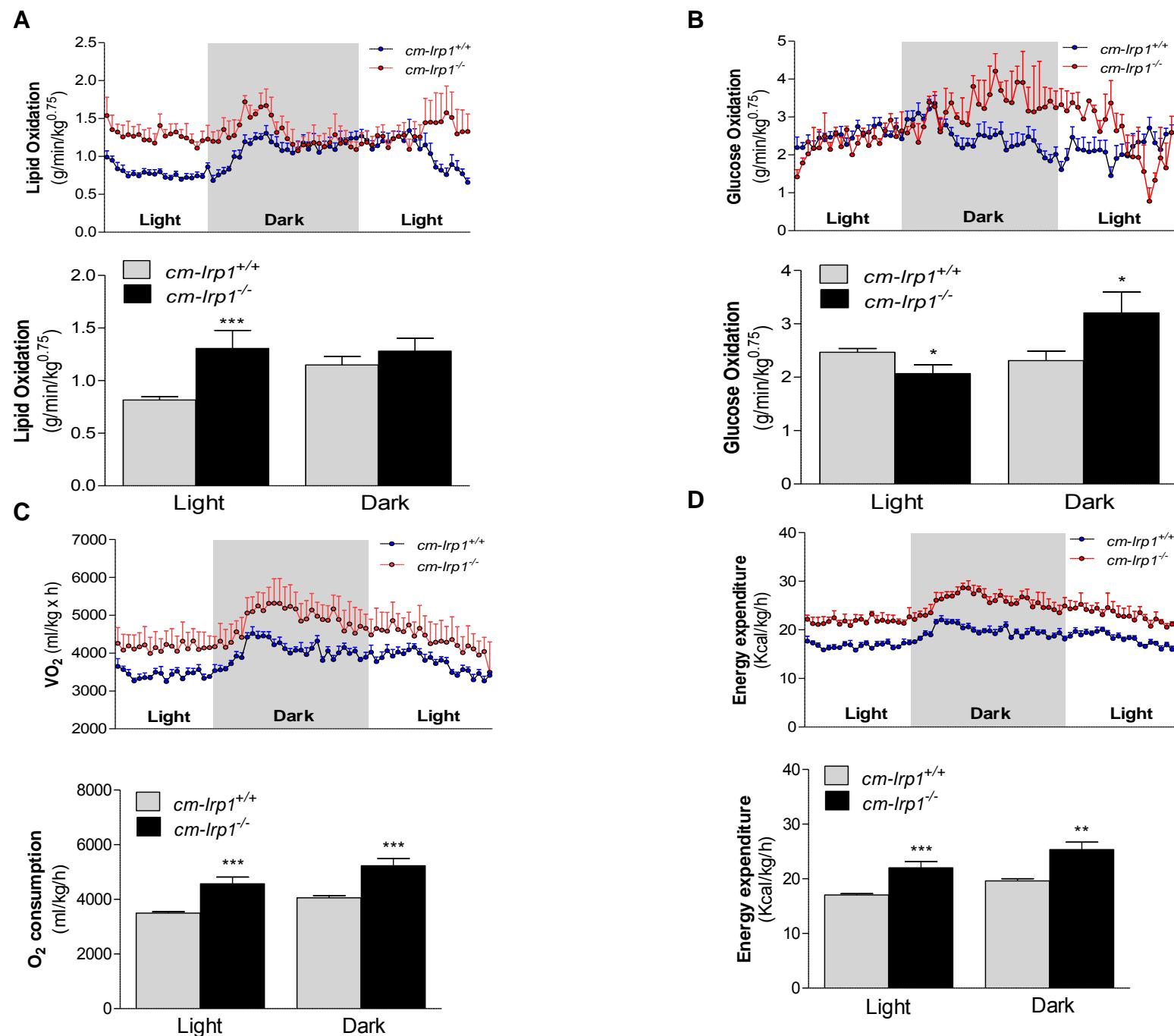


Figure 4

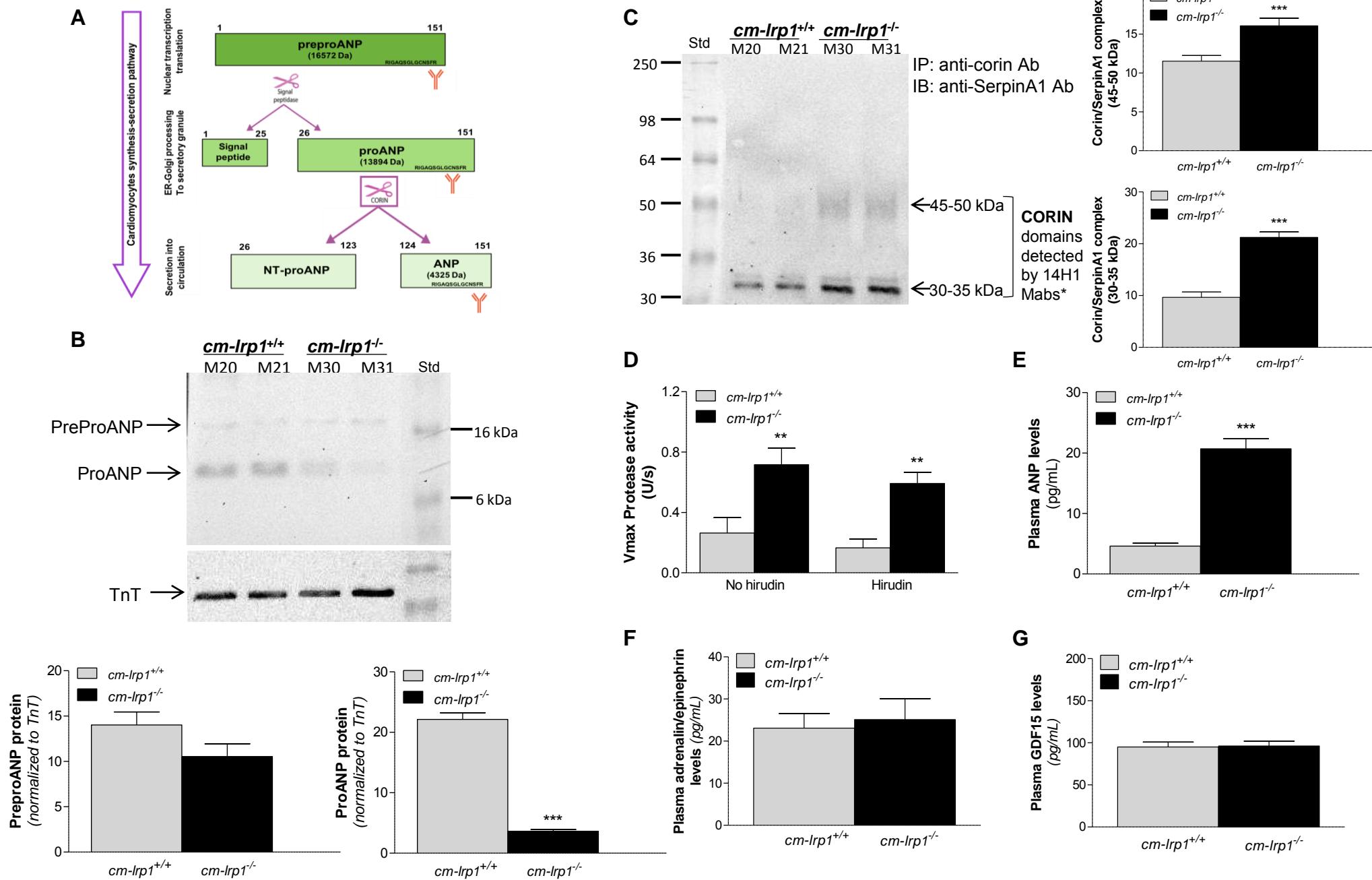
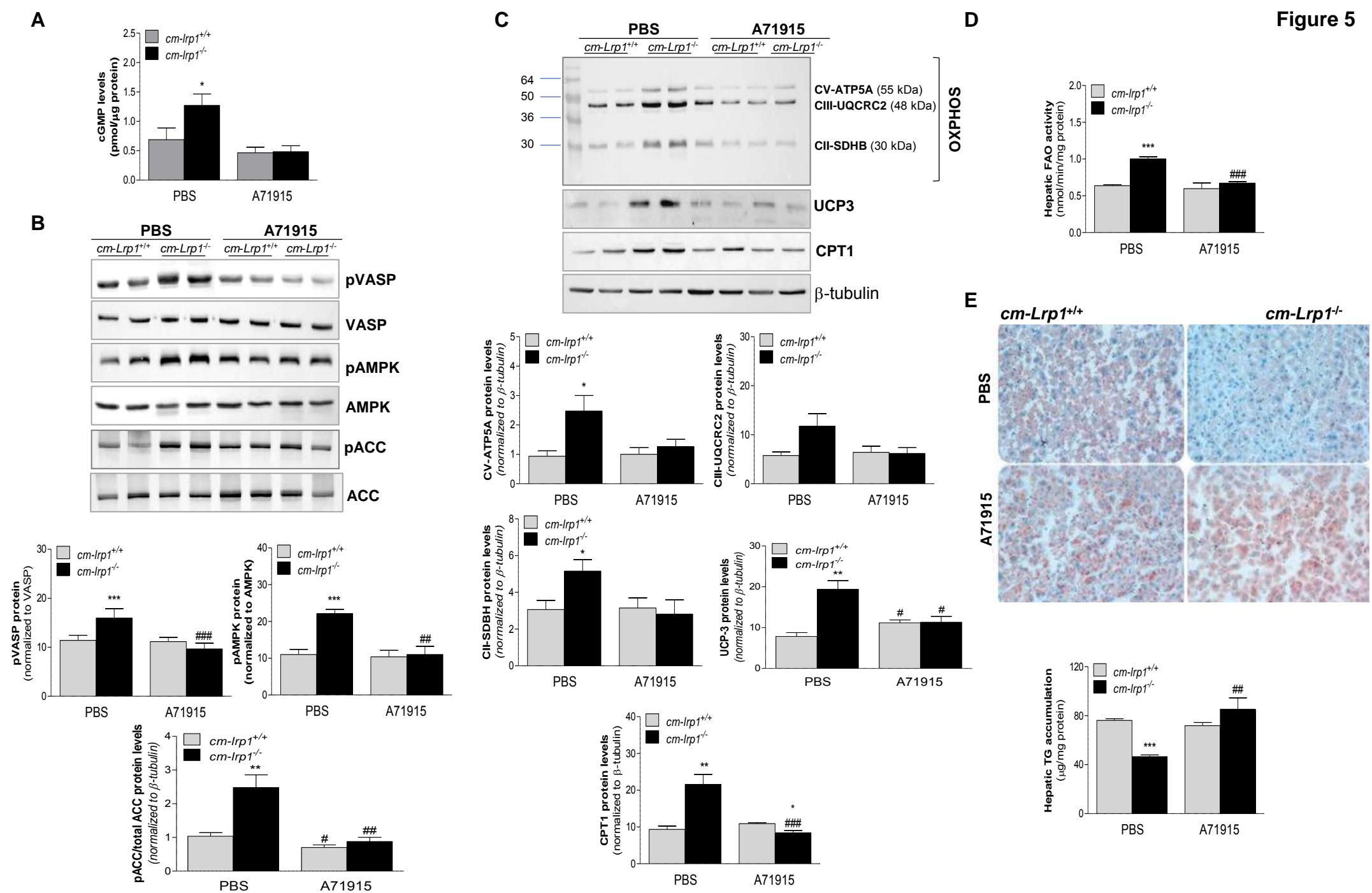


Figure 5



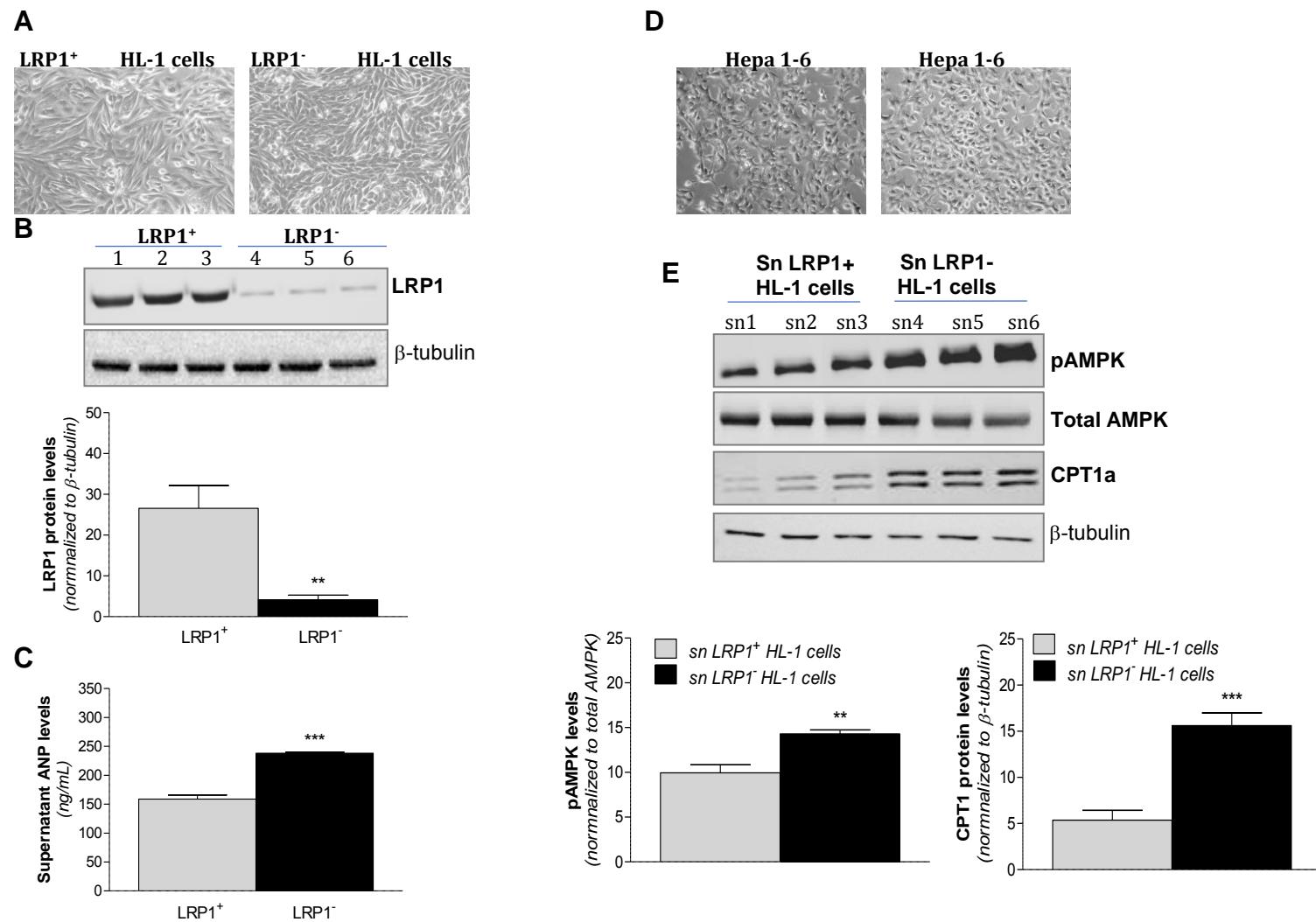


Figure 7

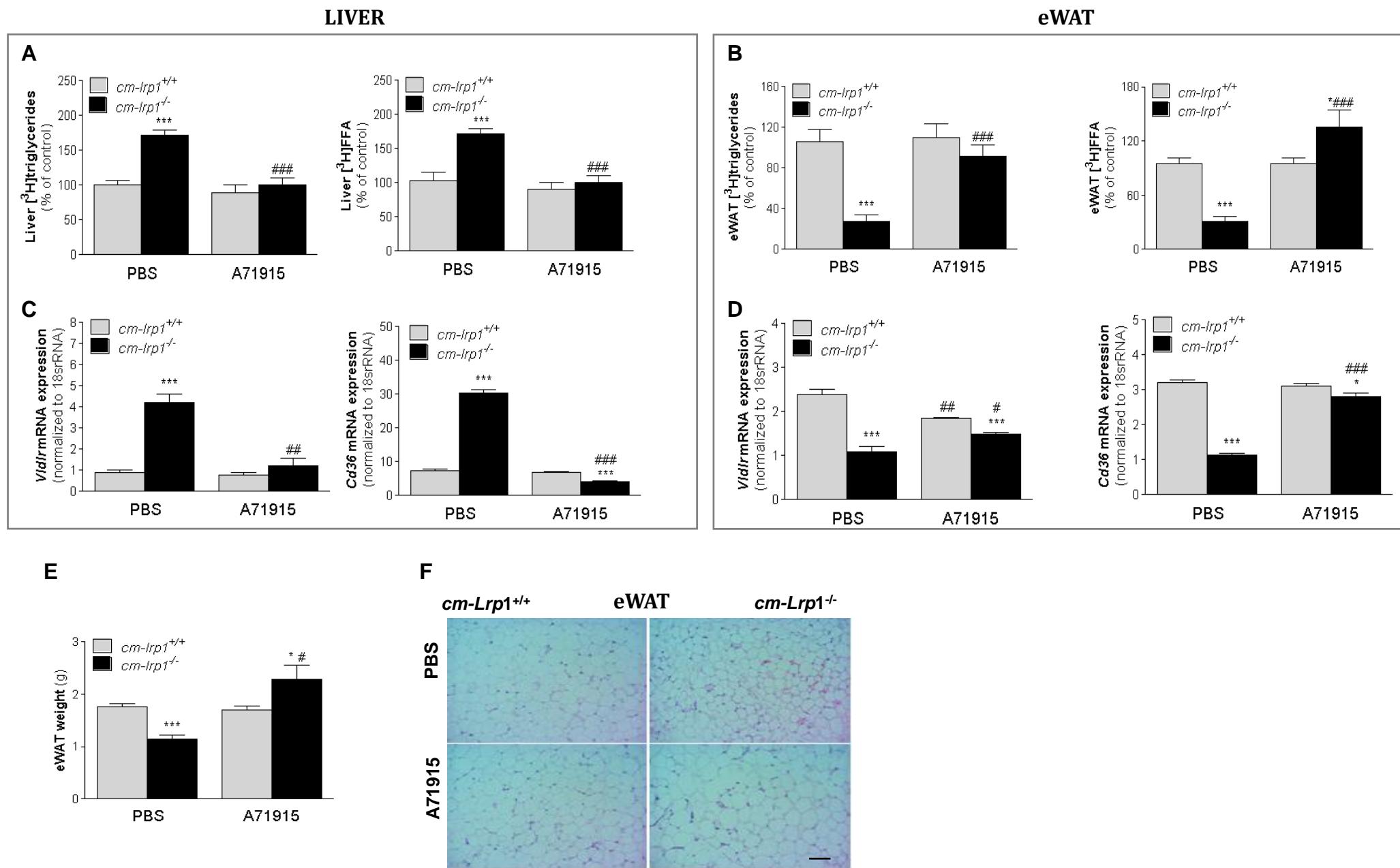


Figure 8

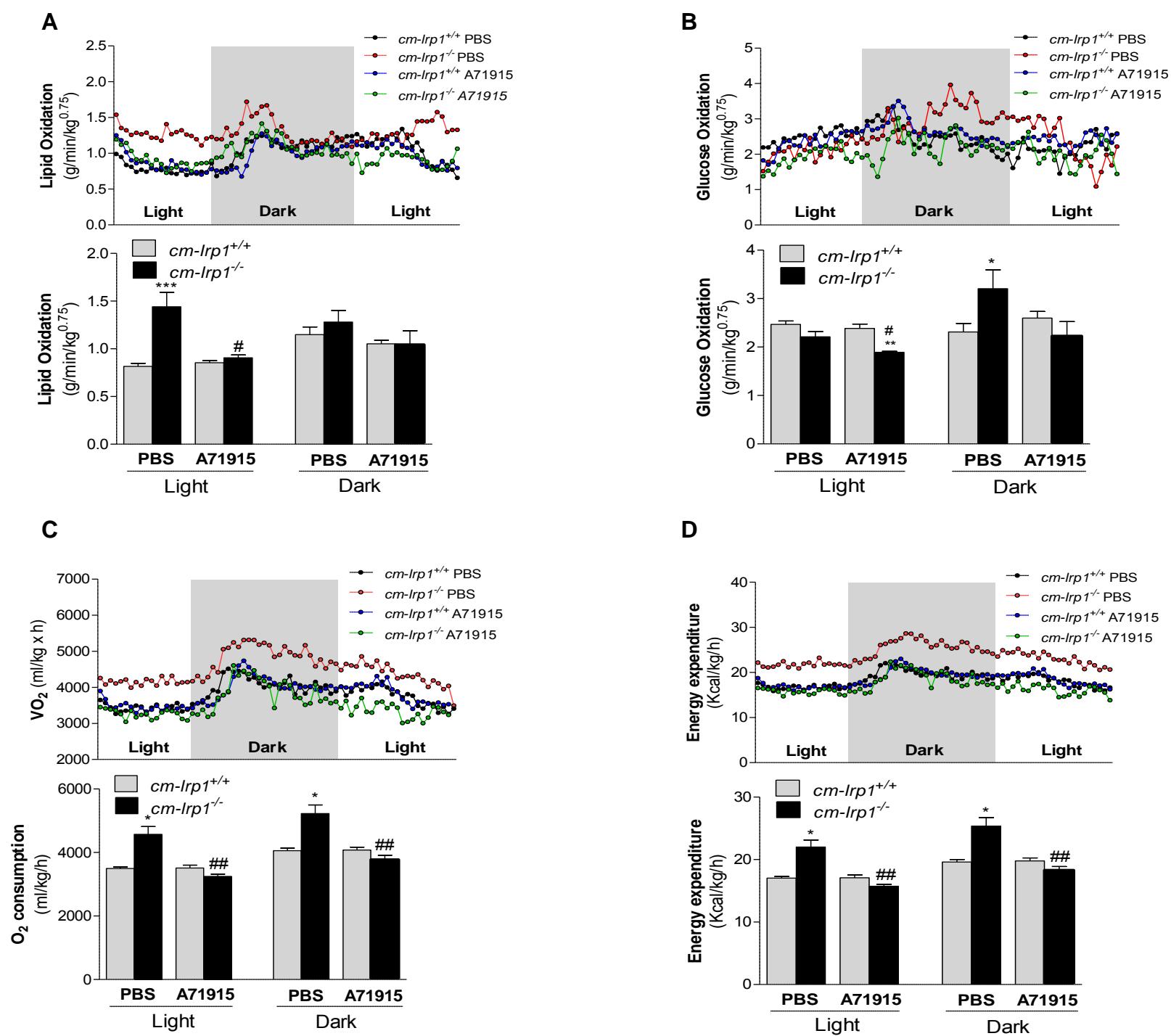
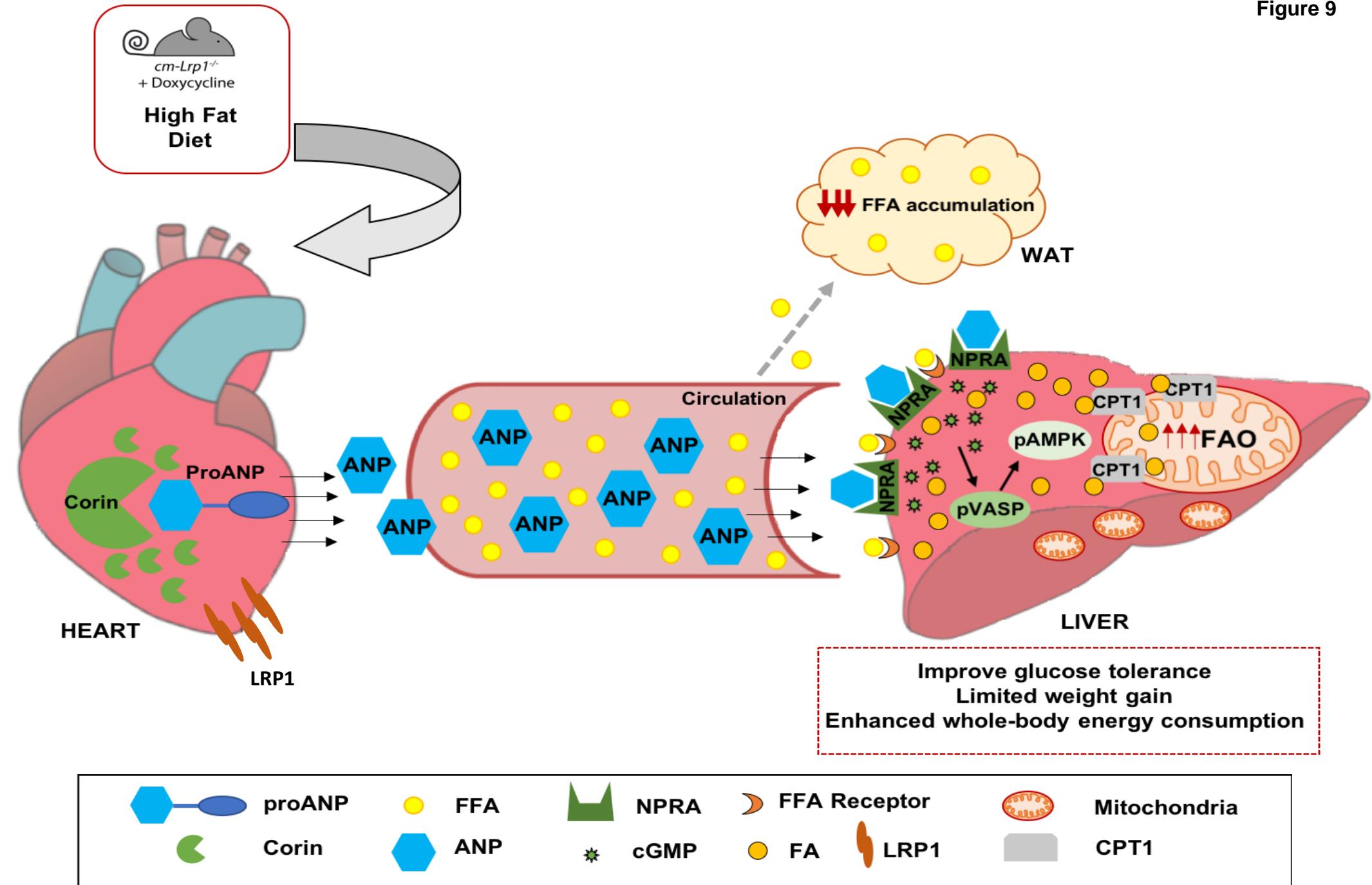


Figure 9



Ms. Ref. No.: METABOLISM-D-19-00958R2

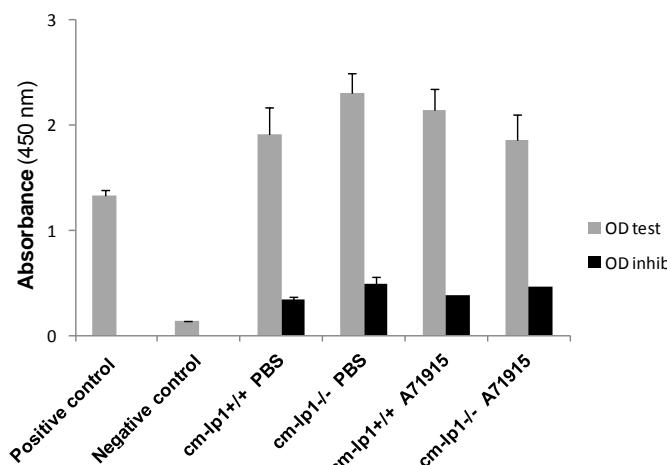
Title: Low-density lipoprotein receptor-related protein 1 deficiency in cardiomyocytes reduces susceptibility to insulin resistance and obesity
Metabolism

Reviewers' comments:

Reviewer #1: The reasoning for why AMPK activity cannot be performed is not convincing. AMPK activity can be measured in a frozen tissue. Authors could still measure AMPK activity in their tissues.

Most of my other concerns were satisfactorily addressed by the authors.

Following the request of Reviewer 1, we have been looking for a suitable ELISA system to measure AMPK activity in frozen tissues. There are not many options that meet this condition except the ELISA with the reference CY-1182 from BioNova. We have performed this ELISA assay with hepatic tissue from the four study II groups. Positive and negative controls were in the optimal range [positive control=1.33±0.05 (optimal>1), and negative control=0.13±0.00 (optimal<0.25)]. However, we did not detect significant differences among the groups (please see the below Figure). This kit offer the possibility to use cells/tissue, but other kinase activities besides that of AMPK are also quantified. Unfortunately, there are not available AMPK-specific inhibitors to solve the nonspecificity of AMPK kinase activity detection of the kit. Dorsomorphin or compound C, the inhibitor recommended in the used ELISA, has been reported to inhibit other kinases much more potently than AMPK and therefore is a non-specific inhibitor (Dasgupta B & Seibel W. *Methods Mol Biol* 2018; 1732: 195-202). The manufacturer recommends to validate the AMPK activity results obtained from the ELISA kit by Western blot analysis to discriminate that there are no other kinases involved. We regret not having currently a system for a reliable detection of AMPK activity other than Western blot.



OD test: Absorbance in the absence of Dorsomorphin

OD inhib: Absorbance in the presence of Dorsomorphin



The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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ITEM	RECOMMENDATION	Section/ Paragraph
Title	1 Provide as accurate and concise a description of the content of the article as possible.	
Abstract	2 Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	
INTRODUCTION		
Background	3 <ol style="list-style-type: none"> Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology. 	
Objectives	4 Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	
METHODS		
Ethical statement	5 Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	
Study design	6 For each experiment, give brief details of the study design including: <ol style="list-style-type: none"> The number of experimental and control groups. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	
Experimental procedures	7 For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: <ol style="list-style-type: none"> How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). When (e.g. time of day). Where (e.g. home cage, laboratory, water maze). Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used). 	
Experimental animals	8 <ol style="list-style-type: none"> Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc. 	

Housing and husbandry	9	<p>Provide details of:</p> <ol style="list-style-type: none"> Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment. 	
Sample size	10	<ol style="list-style-type: none"> Specify the total number of animals used in each experiment, and the number of animals in each experimental group. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. Indicate the number of independent replications of each experiment, if relevant. 	
Allocating animals to experimental groups	11	<ol style="list-style-type: none"> Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. Describe the order in which the animals in the different experimental groups were treated and assessed. 	
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	
Statistical methods	13	<ol style="list-style-type: none"> Provide details of the statistical methods used for each analysis. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). Describe any methods used to assess whether the data met the assumptions of the statistical approach. 	
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	
Numbers analysed	15	<ol style="list-style-type: none"> Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%). If any animals or data were not included in the analysis, explain why. 	
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	
Adverse events	17	<ol style="list-style-type: none"> Give details of all important adverse events in each experimental group. Describe any modifications to the experimental protocols made to reduce adverse events. 	
DISCUSSION			
Interpretation/scientific implications	18	<ol style="list-style-type: none"> Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results². Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research. 	
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	

References:

- Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
- Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

Supplementary Material

[**Click here to download Supplementary Material: 20200214_Supplemental Figures Metabolism R1.pptx**](#)

AUTHOR CONTRIBUTION SECTION

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Data analysis and interpretation: Vicenta Llorente-Cortés, David de Gonzalo-Calvo, Sandra Camino-López, Francisco Blanco Vaca, Antoni Bayes Genis, Antonio Zorzano, Joan Carles Laguna, Dolors Serra, Joan Carles Escola-Gil, David Sebastian.

Writing and review of Manuscript: Elena Revuelta-López, Joan Carles Escola-Gil, Vicenta Llorente-Cortés.