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(54) Title: PEPTIDE AGONISTS OF ADIPONECTIN RECEPTOR 1 AND 2

(57) Abstract: The current invention concerns a peptide capable of binding to adiponectin receptors or 2, characterized in that said peptide has at least 95% sequence identity to an amino acid sequence chosen from SEQ ID 2 to 3 or 5 to 12 as well as the use of such peptides in compositions.



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PEPTIDE AGONISTS OF ADIPONECTIN RECEPTORS 1 AND 2**Technical field**

- 5 The invention pertains to the technical field of agonists of adiponectin receptors 1 and 2 (Adipo R1/2) and their clinical use in subjects.

Background

- 10 The global prevalence of diabetes mellitus is approximately 360 million affected individuals, and is expected to reach 550 million by 2030, or one adult in ten. The prevalence has doubled over the past three decades as a result of obesity epidemic, and poses major health and socio-economic burden. Obesity is nearly invariably associated with insulin resistance in muscle, liver and fat.

- 15 Diabetes is one of the major causes of premature illness and death worldwide due to the severe long-term complications, such as cardiovascular disease, visual disability, renal failure, and neuropathy with limb amputation. Considering that type 2 diabetes (T2D) is increasingly prevalent (about 90% of diabetic patients) and
20 develops early in the patients' life, their life quality and expectancy are notably reduced consequent to severe diabetic complications. Type 1 diabetes (T1D, formerly known as insulin-dependent, juvenile or childhood-onset) is characterized by deficient insulin production, while T2D (previously named non-insulin-dependent or adult-onset) is caused by the inefficient use of insulin by the body's tissues.
25 Although their symptoms may be similar (e.g. polyuria, polydipsia, fatigue), they are less marked in early T2D and explains its late diagnosis, when the complications have already progressed. Being an incurable chronic disease, the costs of medical care are particularly expensive, especially when diabetic complications have developed. Therefore, early diagnosis, prevention and control of diabetes and of its
30 complications greatly contribute to cost savings with patient care.

- Metabolic syndrome (MS) assembles several risk factors that concur in the development of various life-threatening disorders, such as the coronary artery disease, stroke, and T2D. Although the molecular basis of MS remains to be
35 elucidated, all the risk factors are related to obesity. Among the risk factors, the central obesity and insulin resistance are the most important ones.

In addition to its participation to energy homeostasis (i.e., the triglyceride storage and free fatty acid (FFA)/glycerol release), the adipose tissue acts as an endocrine organ that secretes several types of adipokines, such as free fatty acids, adiponectin, adipsin, leptin, plasminogen activator inhibitor-1, resistin, and TNF- α .

5 They may link obesity to MS and the consequent morbid disorders due to their implication in energy and vascular homeostasis, but also in the immune pathways. The pro-inflammatory adipokines are overproduced in obesity, whereas anti-inflammatory or insulin-sensitizing adipokines, such as adiponectin, are decreased. The modulation of the altered production of adipokines may thus have therapeutic
10 potential in the management of MS.

Adiponectin has been reported to improve insulin sensitivity and exert antidiabetic, anti-inflammatory and antiatherogenic effects. Adiponectin binding to its main receptors, AdipoR1 and AdipoR2, triggers the oxidation of FFA and the glucose
15 uptake by skeletal muscle, while liver gluconeogenesis is prevented. AdipoR1 is principally expressed in skeletal muscle, where the signaling pathway of 5' adenosine monophosphate-activated protein kinase (AMPK) is activated. AdipoR2 is mostly expressed in the liver, where it activates the pathway of peroxisome proliferator-activated receptor alpha (PPAR α). The two receptors are also co-
20 expressed in many other cell and tissue types. For instance, pancreatic beta cells express both AdipoR1/R2 and regulate the antiapoptotic effects of adiponectin by activating the MEK-ERK1/2 and PI3K-Akt pathways.

Modulation of adiponectin signaling could have an effect on diseases such as obesity,
25 type 2 diabetes and metabolic syndrome as well as provide a dietary solution.

Sunghwan et al., 2018; WO 2012/142142; Miki Okada-Iwabu et al., 2013 and 2015; and Laszlo Otvos Jr et al., 2011 all describe small molecules able to act as agonists of adiponectin. Sunghwan et al., 2018 and Miki Okada-Iwabu et al., 2013 and 2015
30 discuss the use of these agonists for use in diabetes treatment. However, of these documents, only Sunghwan et al., 2018 is directed to a peptide. While Sunghwan et al., 2018 suggests that the peptide is able to bind to both AdipoR1 and AdipoR2, it is apparent from the specification that binding to AdipoR2 is far less effective.

35 The current invention aims to provide a therapy or solution for at least one of the problems mentioned above, and which has a broad spectrum.

Summary of the invention

The present invention provides for peptides according to claim 1 or a molecule according to claim 7. These peptides or molecules are capable of binding to AdipoR1 and AdipoR2 and are agonists of AdipoR1 and AdipoR2.

In a second aspect, the current invention also relates to within AdipoR1 and AdipoR2, wherein said region has an amino acid sequence according to SEQ ID n°13. In a third aspect, the current invention provides a composition according to claim 9.

Finally, the current invention provides for a use of the peptides or compositions as described above in a subject, preferably a human.

Detailed description of the invention

The present invention concerns peptides capable of binding to AdipoR1 and AdipoR2 and their (therapeutic) use.

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the present invention.

As used herein, the following terms have the following meanings:

"A", "an", and "the" as used herein refers to both singular and plural referents unless the context clearly dictates otherwise. By way of example, "a compartment" refers to one or more than one compartment.

"About" as used herein referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/- 20% or less, preferably +/-10% or less, more preferably +/-5% or less, even more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, in so far such variations are appropriate to perform in the disclosed invention. However, it is to be understood that the value to which the modifier "about" refers is itself also specifically disclosed.

"Comprise", "comprising", and "comprises" and "comprised of" as used herein are synonymous with "include", "including", "includes" or "contain", "containing", "contains" and are inclusive or open-ended terms that specifies the presence of what
5 follows e.g. component and do not exclude or preclude the presence of additional, non-recited components, features, element, members, steps, known in the art or disclosed therein.

The recitation of numerical ranges by endpoints includes all numbers and fractions
10 subsumed within that range, as well as the recited endpoints.

The expression "% by weight", "weight percent", "%wt" or "wt%", here and throughout the description unless otherwise defined, refers to the relative weight of the respective component based on the overall weight of the formulation.

15 In a first aspect, the current invention provides a peptide capable of binding to adiponectin receptors 1 or 2.

The term "peptide," as used herein and in the appended claims, refers to any compound containing two or more amino acid residues joined by an amide bond
20 formed from the carboxyl group of one amino acid residue and the amino group of the adjacent amino acid residue. The amino acid residues may have the L-form as well as the D-form, and may be naturally occurring or synthetic, linear as well as cyclic. Also included within the term "peptide" as used herein and in the claims are polypeptides and peptide dimers which can be peptides linked C-terminus to N-terminus (tandem repeats) or peptides linked C-terminus to C-terminus or N-terminus to N-terminus (parallel repeats).
25

By preference, said peptide is a synthetic peptide. By the term "synthetic peptide" it is understood to refer to a peptide which has been artificially synthesized. Such synthesis methodologies are readily known in the art. The term "therapeutic
30 peptide" as used herein denotes a bioactive peptide that has therapeutic utility.

Another method for the preparation of the peptides according to embodiments disclosed herein is the use of peptide mimetics. Mimetics are peptide-like molecules which mimic elements of protein or peptide secondary structure. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins
35 exists chiefly to orient amino acid side chains in such a way as to facilitate molecular

interactions. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

The peptide according to the embodiments of the current invention may be purified. Generally, "purified" will refer to a protein or peptide composition which has been
5 subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this will refer to a composition in which the peptide forms the major component of the composition, such as constituting about 50% or more of the peptides in the composition.

10 Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. Actual
15 units used to represent activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation;
20 chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method
25 for the preparation of a substantially purified protein or peptide.

There is no general requirement that the peptide always be provided in the most purified state. It is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same
30 general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater - fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the
35 activity of an expressed protein.

In an embodiment, the peptides will have a length of 6 to 25 amino acids, more preferably between 10 to 15 amino acids, even more preferably twelve amino acids.

- In another or further embodiment, said peptides according to the current invention share at least 95%, more preferably at least 99% sequence identity to an amino acid sequence chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 4, 5 or SEQ:ID n°12. In another embodiment, said peptides have a sequence which differs maximally 3, more preferably maximally 2, even more preferably maximally 1 amino acid from one of the sequences chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 4, 5 or SEQ:ID n°12.
- 10 In another embodiment, said peptides have an amino acid sequence which incorporates one of the amino acid sequences chosen from SEQ:ID n° 1 to 12, or a sequence which has at least 95%, more preferably 99% sequence identity with the amino acid sequences chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 4, 5 or SEQ:ID n°12.
- 15 The term "sequence identity" as used herein refers to the extent that sequences are identical on an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid occurs in both sequences to yield the number of
- 20 matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. A gap, i.e., a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. Determining the percentage of
- 25 sequence identity can be done manually, or by making use of computer programs that are available in the art. Examples of useful algorithms are PILEUP. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).
- 30 Amino acid sequence variants of a peptide contemplated herein may be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the peptide which may not be critical for function. Substitutional variants typically contain an alternative amino acid at one or more sites within the peptide and may be designed to modulate one or more properties of the polypeptide such
- 35 as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar size and side chain or

functional group. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to glycine, valine or leucine; arginine to lysine; asparagine to glutamine; aspartate to glutamate; cysteine to methionine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to glutamine, tyrosine, arginine, lysine, asparagine or cysteine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to phenylalanine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

10

In another embodiment, said peptides have an amino acid sequence which differs by maximal 3, maximal 2, more preferably maximal 1 amino acid from one of the amino acid sequences chosen from SEQ:ID n° 1 to 12.

15 Said peptides have a half-life of between 1 and 30 hours, more preferably between 2 and 20 hours.

The current invention equally relates to a synthetic sequence encoding for a peptide according to SEQ ID n° 1 – 12 and according to one of the embodiment above.

20 AdipoR1 and AdipoR2 present 66.41% sequence identity and are composed of 7 transmembrane domains with a membrane topology that is reversed when compared to the G-protein-coupled receptors (GPCRs), meaning that their N-terminus is internal, whereas the C-terminus is external.

25 Within the C-terminal domain of adiponectin receptors (AdipoR), a 12-amino acid sequence (AdipoR-12C) was identified that is homologous in AdipoR1 (Q96A54) and AdipoR2 (Q86V24), both in humans and mice.

30 The peptides as described above are able to bind to a (C-terminal) region of adiponectin receptors 1 or 2, wherein said region comprises an amino acid sequence according to SEQ ID 13. In a further preferred embodiment, said peptides are able to bind to the amino acid sequence as given in SEQ ID n° 13 and which is part of the adiponectin receptors 1 or 2.

35 The current invention is thus also directed to the sequence given in SEQ ID n° 13 which is conserved in both AdipoR1 and AdipoR2 and to molecules, including

peptides (such as the peptides as identified above), proteins such as antibodies or compounds such as small molecules able to bind to this conserved region. For the purpose of the current invention, the term "small molecule" is defined as a usually low molecular weight (< 900 daltons) organic compound that may regulate a biological process, with a size on the order of 1 nm. Said small molecule is a molecule able to bind specific biological macromolecules (in the current case AdipoR1 and AdipoR2) and acts as an effector, altering the activity or function of the target.

Said binding molecules may be able to block (antagonist or inhibitors) or activate (agonists) the adiponectin receptor signaling. By preference, said binding molecules are agonists of AdipoR1 and AdipoR2 and as such are able to activate AdipoR1 and/or AdipoR2 signaling upon binding and thus able to modulate the lipid and glucose metabolism of a subject, preferably a mammal, such as a human.

Suitable molecules can be identified via ligand binding assays, phage display, computational modeling or other high throughput screenings.

The present invention also pertains to a composition, preferably a pharmaceutical composition comprising one or more peptides or molecules able to bind to SEQ ID n° 13 according the embodiments as described above.

Embodiments herein provide for administration of compositions to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the active agent (e.g., pharmaceutical chemical, protein, gene, antibody, aptamers etc. of the embodiments) to be administered in which any toxic effects are outweighed by the diagnostic or therapeutic effects of the active agent. Administration of an active amount of the compositions according to the current invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result, whether it may be for imaging or therapeutic reasons.

For example, an active amount of a compound may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptides to elicit a desired response in the individual. Dosage regimen may be adjusted to provide the optimum therapeutic response.

Compositions containing a peptide of the current invention, or analog thereof, or a functional derivative thereof (e.g., a mimetic of said peptides) or a molecule able to bind to SEQ ID n° 13 may be administered to a subject, for example by subcutaneous, intravenous, peritoneal, intracardiac, intracoronary, intramuscular, by oral administration (formulated as solutions, liquids, (lyophilized) powders, capsules, tablets, liposomes, and the like), inhalation, transdermal application, intravaginal application, topical application, intranasal or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the active agent from the degradation by enzymes, acids and other natural conditions that may inactivate the compound. In a preferred embodiment, the composition may be administered intravenously.

The active agent (in the current case the peptide) may be administered to a subject in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. The term "pharmaceutically acceptable carrier" as used herein is intended to include diluents such as saline and aqueous buffer solutions. It may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. The active agent may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. In another embodiment, the peptides could be loaded in polymeric microparticles of chitosan, dextran, alginate, PLGA etc., in colon-targeted microparticles, hydrogel-based microparticles, or in microcapsules or microspheres often composed of polymers. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use may be administered by means known in the art. For example, sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion may be used.

Sterile injectable solutions can be prepared by incorporating the active agent in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

Aqueous compositions can include an effective amount of the active agent, being one or more peptides according to the current invention dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Compounds and biological materials disclosed herein can be purified by means known in the art. Solutions of

the active compounds as free-base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In an embodiment, said composition may be formulated to be administered via a medical device such as an insulin pump. Said composition could be either separately administered or in combination with insulin. Other medical devices are insulin syringes or insulin pens.

Active agents may be formulated within a mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 5.0 mg per dose or even about 1 to 10 grams per dose, depending on the specific reasons for use (e.g. therapeutic or dietary). If the peptide or molecule is used for therapeutic reasons the chosen dose may lie between 1 mg/dose to 15 g/dose, more preferably 1 g to 10 g/dose.

When the composition is used for therapeutic purpose, a single dose or multiple doses can also be administered on an appropriate schedule for a predetermined condition such as daily, bi-weekly, weekly, bimonthly etc. Pharmaceutical compositions are administered in an amount, and with a frequency, that is effective to modulate side effects. The precise dosage and duration of treatment may be determined empirically using known testing protocols or by testing the compositions in model systems known in the art and extrapolating therefrom. Dosages may also vary with the severity of the condition. In certain embodiments, the composition range can be between 10 and 75 mg/kg introduced daily or weekly to a subject. A therapeutically effective amount can be also measured in molar concentrations and can range between about 10 nmol to about 100 μ mol of peptide/molecule per kg body weight of said subject, more preferably between 20 nmol to 50 μ mol of peptide/molecule per kg body weight of said subject, even more preferably between 1 μ mol and 40 μ mol of peptide/molecule per kg body weight of said subject.

Therapeutic application of the peptides, molecules and compositions according to the current invention comprising them can be accomplished by any suitable therapeutic method and technique presently or prospectively known to those skilled in the art. Further, the peptides or molecules of the invention can be used as starting

materials or intermediates for the preparation of other useful compounds and compositions.

In a particular aspect of the current invention, the peptides, molecules or compositions as described above can be used for therapeutic use or as a food supplement, preferably a dietary food supplement.

As explained, adiponectin signaling pathways via AdipoR1 and AdipoR2 regulate glucose and lipid metabolism, showing a relevance for T2D and obesity associated pathologies. The peptides according to the current invention and other molecules able to bind to the sequence given in SEQ ID n°13 of AdipoR1 and AdipoR2 are shown to be able to influence the clinical picture of diseases or syndromes linked to adiponectin signaling. The current invention provides thus a method for treating or controlling syndromes or diseases linked to adiponectin signaling through AdipoR1 and AdipoR2. Examples are for instance pathologies such as obesity, MS, cardiovascular diseases (CVD), fatty liver disease, liver fibrosis, Type 2 Diabetes or systemic disease. This will be further elaborated on in the experimental data section.

The present invention will be now described in more details, referring to examples that are not limitative.

Description of figures

Figure 1 shows the location of the targeted AdipoR-12C (white frame) at the end of the 7th transmembrane domain (TMD7) that precedes the C-terminal domain (CTD) in AdipoR1 (PDB id: 3WXV) (A, B) and AdipoR2 (PDB id: 3WXW) (C, D). The specific affinity (expressed as target/BSA) of the phage pools recovered after three rounds of panning was assessed against AdipoR-12C (E) and AdipoR1 (F), whereas that of the 50 phage clones isolated from the 3rd round phage pool was evaluated against AdipoR-12C (G); the gray horizontal line represents the mean specific affinity.

Figure 2 shows the amino acid frequency in the sequence of 12 identified peptides (A) and titration curves with K_d values of the three selected peptide clones (B).

Figure 3 shows the three-dimensional structure (A) and spatial conformation (B) of peptides P16, P17 and P18, compared to AdipoR1-12C and AdipoR2-12C. The three-dimensional structure of peptides was drawn with ACD/ChemSketch 2.0 software.

The three-dimensional structure of AdipoR1-12C and AdipoR2-12C and the spatial conformations of all molecules were obtained with MarvinSketch 5.11.5 software (2013, <http://www.chemaxon.com>).

5 **Figure 4** shows colocalization of peptides P16, P17 and P18 with mouse AdipoR1 in muscle (A) and AdipoR2 in liver (B) as observed by immunofluorescence. AdipoR1 and AdipoR2 are detected with fluorescein, peptides are evidenced with Texas Red, whereas nuclei are revealed with DAPI. The Manders' Colocalization Coefficients M1 (overlap of channel 1 over channel 2) and M2 (overlap of channel 2 over channel 1)
10 were evaluated using the JACoP plugin of ImageJ software.

Figure 5 shows colocalization of peptides P16, P17 and P18 with AdipoR1 (A) and insulin (B) in human pancreas as observed by immunofluorescence. AdipoR1 is detected with fluorescein, insulin observed with Texas Red, whereas nuclei are
15 revealed with DAPI. Peptides are stained with Texas Red in (A) and with fluorescein in (B). The Manders' Colocalization Coefficients M1 (overlap of channel 1 over channel 2) and M2 (overlap of channel 2 over channel 1) were evaluated using the JACoP plugin of ImageJ software.

20 **Figure 6** shows colocalization of slow myosin with AdipoR1 (A) and peptides P16, P17 and P18 (B) in mouse muscle as observed by immunofluorescence. Slow myosin is stained with Texas Red, peptides are revealed with fluorescein, whereas nuclei are detected with DAPI.

25 **Figure 7** shows expression of AdipoR2 by HepaRG cells (A) and of AdipoR1 by differentiated C2C12 cells (B) observed by immunofluorescence with fluorescein; nuclei are stained with DAPI. The cells were induced for 15, 60 and 135 minutes by incubation in a culture medium enriched in glucose (Glc, 25 mM for HepaRG; 39 mM for C2C12) or glucose and free fatty acids and cholesterol (FFAC). The relative ratio
30 of fluorescent labeling (RRFL, normalized to cell number and the background) of AdipoR2 (left) and AdipoR1 (right) was evaluated with ImageJ software and represented graphically in (C). The results are expressed as means $\pm$ SD.

Figure 8 shows the effect of peptides and of a commercial AdipoR agonist
35 (AgoAdipoR) on AMPK activation by T172 phosphorylation (AMPK α -pT172) in HepaRG (A, B, C, D) and C2C12 cells (E, F, G, H). The cells were incubated for 15, 60 and 135 minutes in a culture medium containing a glucose supplement (A, C, E, G) or the basal glucose concentration (B, D, F, H). For each culture condition, the

medium was supplemented (C, D, G, H) or not (A, B, E, F) with a mixture of free fatty acids and cholesterol (FFAC). The results are expressed as means $\pm$ SD. The statistical significance of the results was calculated by ANOVA, using the SigmaPlot 11.0 software, by comparing each test group to the control group, incubated in the same culture media as the test groups excepting the peptides and AgoAdipoR. NS stands for non-significant. The peptides that present a significant effect are framed with the same color as their graphical representation.

Figure 9 shows the effect of peptide P17 on SDHA and GK expression in HepaRG (A) and differentiated C2C12 (B) cells incubated for 60 minutes in a culture medium enriched with glucose (Glc, 25 mM for HepaRG; 39 mM for C2C12) or glucose and free fatty acids and cholesterol (FFAC). SDHA was detected with Texas Red, GK was observed with fluorescein, while nuclei were stained with DAPI. The relative ratio of fluorescent labeling (RRFL, normalized to cell number and the background) was evaluated with ImageJ software and represented graphically in (C). The results are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 10 shows the relative ratio of fluorescent labeling (RRFL, normalized to cell number and the background) of the microphotographs of AdipoR1 colocalization with lysosomes and caveolae (examples shown in Figures 11 and 12) was evaluated with ImageJ software and represented graphically. The results are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 11 shows colocalization of AdipoR1 with lysosomes (observed with anti-LAMP1 antibody) (A) and caveolae (observed with anti-caveolin-1 antibody) (B) in differentiated C2C12 cells incubated for 60 minutes in a culture medium enriched with glucose (Glc, 39 mM) and which were stimulated or not with peptide P17; plain control cells were incubated in basal culture medium. AdipoR1 was detected with Dylight 594, lysosomes and caveolae were observed with Dylight 488, while nuclei were stained with DAPI. The Manders' Colocalization Coefficients M1 (overlap of channel 1 over channel 2) and M2 (overlap of channel 2 over channel 1) were evaluated using the JACoP plugin of ImageJ software.

Figure 12 shows colocalization of AdipoR1 with lysosomes (observed with anti-LAMP1 antibody) (A) and caveolae (observed with anti-caveolin-1 antibody) (B) in differentiated C2C12 cells incubated for 60 minutes in a culture medium enriched

with glucose (Glc, 39 mM) and free fatty acids and cholesterol (FFAC) and which were stimulated or not with peptide P17; plain control cells were incubated in basal culture medium. AdipoR1 was detected with Dylight 594, lysosomes and caveolae were observed with Dylight 488, while nuclei were stained with DAPI. The Manders' Colocalization Coefficients M1 (overlap of channel 1 over channel 2) and M2 (overlap of channel 2 over channel 1) were evaluated using the JACoP plugin of ImageJ software.

Figure 13 shows the effect of peptide P17 on the body weight of db/db mice fed on a Western high-fat diet containing 60 kcal % or on a standard chow diet containing 10 kcal %. The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice fed on a standard chow diet, which did not receive any treatment. The results are shown in box-and-whisker plots and the statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 14 shows the effect of peptide P17 on glycemia (A), plasma triglycerides and adiponectin (B) of db/db mice fed on a Western high-fat diet containing 60 kcal % or on a standard chow diet containing 10 kcal %. The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice fed on a standard chow diet, which did not receive any treatment. The results of glycemia are shown in box-and-whisker plots. Plasma triglycerides and adiponectin are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 15 shows the effect of peptide P17 on the expression of AdipoR1 in skeletal muscle (A) and of AdipoR2 in liver (B) of db/db mice fed on a Western high-fat diet containing 60 kcal % or on a standard chow diet containing 10 kcal %. Phosphorylated AMPK (AMPK-p) in skeletal muscle (C) and liver (D) and phosphorylated PPAR α (PPAR α -p) in liver (E) were also analyzed on tissue samples of the same mice. The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice (F) fed on a standard chow diet, which did not receive any treatment. The different biomarkers are observed by immunofluorescence with fluorescein (AdipoR1, AdipoR2, PPAR α) or with Dylight 488 (AMPK-p); nuclei are stained with DAPI.

Figure 16 shows the relative ratio of fluorescent labeling (RRFL, normalized to cell number and the background) evaluated on microphotographs of the same experimental conditions as in Figure 15 using ImageJ software and the results were represented graphically in (A) for AdipoR1 in muscle, in (B) for AdipoR2 in liver, in (C) for AMPK-pT172 in muscle, in (D) for AMPK-pT172 in liver, and in (E) for PPAR α -pS12 in liver. The results are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 17 shows the effect of peptide P17 on liver apoptosis of db/db mice fed on a Western high-fat diet containing 60 kcal % or on a standard chow diet containing 10 kcal %. The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice fed on a standard chow diet, which did not receive any treatment. Apoptotic livers from Balb/c mice treated with anti-Fas antibody were used as positive controls. Apoptosis was observed by the immunofluorescent detection of activated caspase-3, stained with Dylight 488; nuclei are stained with DAPI (A). The relative ratio of fluorescent labeling (RRFL, normalized to cell number and the background) was evaluated on microphotographs and the results were represented graphically in (B). The results are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Figure 18 shows the Masson's trichrome staining of liver tissue from db/db mice fed on a Western high-fat diet containing 60 kcal % or on a standard chow diet containing 10 kcal %. The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice fed on a standard chow diet, which did not receive any treatment. Apoptotic livers from Balb/c mice treated with anti-Fas antibody were used as positive controls.

Figure 19 shows the effect of peptide P17 on pancreatic islet cell apoptosis of db/db mice fed on a Western high-fat diet containing 60 kcal % (A) or on a standard chow diet containing 10 kcal % (B). The mice were treated with P17 each evening for a duration of 4 days and were compared to control db/db mice treated with PBS and fed on the same diet or with healthy NMRI mice fed on a standard chow diet, which did not receive any treatment (C). Apoptosis was observed by the immunofluorescent detection of activated caspase-3, stained with Dylight 488. Pancreatic beta cells were detected by the immunofluorescent staining of insulin, observed red with Texas Red; nuclei were stained with DAPI.

Figure 20 shows the colocalisation of activated caspase-3 with pancreatic alpha cells detected by the immunostaining of glucagon. Apoptosis was observed by the immunofluorescent detection of activated caspase-3, stained with Dylight 488.

5 Pancreatic alpha cells were observed with Texas Red; nuclei were stained with DAPI (A). The percentage of beta cells and alpha cells was related to the total number of beta and alpha cells per pancreatic islet; the total number of beta cells per pancreatic islet is also represented (B). The results are expressed as means $\pm$ SD. The statistical differences were calculated by ANOVA, using the SigmaPlot 11.0 software.

Experimental data

Materials and Methods

15 1. Targeting of AdipoR-12C by phage display and phage clone characterization

1.1. Phage display panning and DNA sequencing of the selected phage clones

Cys was coupled to the N-terminus of AdipoR-12C via a polyethylene glycol (PEG) spacer, whereas its C-terminus was amidated. Cys was used to immobilize AdipoR-12C on the surface of magnetic beads (Dynabeads[®] M280 Tosylactivated, Life Technologies, Gent, Belgium) according to the manufacturer instructions. AdipoR-12C was synthesized by the PolyPeptide company (Strasbourg, France) and presented the following composition: Cys-8-amino-3,6-dioxanooctanoyl-His-Phe-Tyr-Gly-Val-Ser-Asn-Leu-Gln-Glu-Phe-Arg-CONH₂.

AdipoR-12C was screened with a combinatorial linear 12-mer peptide library fused to the minor coat protein (pIII) of M13 bacteriophage (PhD-12, New England BioLabs Inc., Bioké, Leiden, The Netherlands). The *Escherichia coli* host ER2738 (*E. coli* K12 ER2738, F+, tetracycline-resistant strain; New England BioLabs) was employed for phage amplification and clone isolation.

Bovine serum albumin (BSA) was used as a control protein during the preselection steps of the panning rounds to exclude non-specific phages. To immobilize BSA on Dynabeads, its disulfide bonds were reduced with Tris [2-carboxyethyl] phosphine hydrochloride (TCEP) reagent (Immobilized TCEP Disulfide Reducing Resin, Thermo Fisher Scientific, Erembodegem, Aalst, Belgium) according to the manufacturer instructions. Both AdipoR-12C and BSA coupled Dynabeads were blocked for 1h with the blocking buffer (0.5% BSA in sodium Phosphate Buffered Saline

(NaPBS, for 1L): 0.262 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.901 g $\text{Na}_2\text{HPO}_4 \cdot 10 \text{H}_2\text{O}$, 0.88 g NaCl, pH 7.4). To increase the specificity and stringency of the selected phage clones, the incubation time with the target (at an estimated concentration of 102 μM) was reduced stepwise during the 3 panning rounds (120, 90 and 60 min); the incubation times with BSA were increased stepwise (60, 90, 120 min), while the Tween-20 concentration was increased at each panning round from 0.1% to 0.5% in the incubation and rinsing buffer.

The DNA of the selected phage clones was isolated and purified by phenol extraction – ethanol precipitation, and it was sequenced by the company Beckman Coulter Genomics (Grenoble, France). The DNA sequences and the encoded peptides were read with JaMBW 1.1 software (<http://bioinformatics.org/JaMBW/>). Peptide sequences were aligned with pertinent proteins by BLAST (The Basic Local Alignment Search Tool).

1.2. Binding of phages to AdipoR-12C and AdipoR1

To evaluate the phage binding to AdipoR-12C, this last one was immobilized (50 $\mu\text{g}/\text{mL}$ in NaPBS containing 10 mM EDTA, pH 7.2; 150 $\mu\text{L}/\text{well}$) in the wells of a Pierce® maleimide activated 96-well ELISA plate (Thermo Fisher Scientific) according to the manufacturer instructions. The control wells were coated with BSA (50 $\mu\text{g}/\text{mL}$), with disulfide bonds reduced as described above. After 2.5 hours of incubation, the plate was rinsed with NaPBS pH 7.2 (0.05% Tween-20) and then blocked with 10 $\mu\text{g}/\text{mL}$ of cysteine-HCl (Thermo Fisher Scientific) prepared in the same buffer as AdipoR-12C and incubated (200 $\mu\text{L}/\text{well}$) for 1h at room temperature (RT). After rinsing, the phage samples (5x10¹¹/120 μL of NaPBS pH 7.2, 0.05% Tween-20) were incubated with AdipoR-12C or BSA coated wells for 1h at RT. The plate was then rinsed, and bound phages were detected with HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) diluted 1:5000 in NaPBS pH 7.2, containing 5 mg BSA/mL. The staining reaction was developed with ABTS [2,2'-Azino-bis(3-Ethylbenzothiazoline-6-sulfonic acid), diamonium salt (Sigma-Aldrich, Bornem, Belgium)] solution completed with 0.05% H_2O_2 . The OD₄₀₅ was measured using a microplate reader (StatFax-2100, Awareness Technology, Fisher Bioblock Scientific, Tournai, Belgium).

The binding to human AdipoR1 (Origene Technologies, Sanbio B.V., Uden, The Netherlands) was evaluated after protein immobilization (10 $\mu\text{g}/\text{mL}$, NaHCO_3 0.1 M, pH 8.6; 100 $\mu\text{L}/\text{well}$; 4°C overnight) in the wells of a medium binding Microlon® ELISA plate (Greiner Bi-One, Wemmel, Belgium). After blocking the wells with blocking buffer (0.5% BSA in NaHCO_3 0.1 M, pH 8.6; 300 $\mu\text{L}/\text{well}$; 2h, 4°C),

the wells were rinsed (rinsing/incubation buffer: NaPBS pH 7.2, 0.5% Tween-20) and then incubated (2h, RT, mild agitation at 350 rpm) with phages diluted in the rinsing/incubation buffer at 5×10^{11} virions/100 μ L, or a range of concentrations (10^{12} to 2×10^9 virions/100 μ L) for the estimation of the apparent dissociation constant (K_d^*): the phage binding to AdipoR1 (test wells) and to BSA (control wells) was assessed concomitantly. The wells were then rinsed again, and the bound phages were detected as described above.

2. In vitro evaluation of the selected AdipoR-binding peptides

Based on the affinity tests, the peptides were synthesized (PolyPeptide Laboratories) as biotinylated or not biotinylated 8-amino-3,6-dioxaoctanoyl derivatives.

2.1. Binding of peptides to AdipoR1/R2 expressed by mouse muscle and liver and by human pancreas

The binding of peptides to healthy mouse (NMRI mice, mean body weight of 23g, Harlan Laboratories, Horst, The Netherlands) skeletal muscle and liver, and to human pancreas (provided by the Pathology Department of the Erasme Hospital, ULB, Brussels, Belgium) was evaluated by immunofluorescence, the same as the colocalization of peptides with AdipoR1/R2. The biopsies were collected after written informed consent. The patient samples of human pancreas were collected retrospectively from the records of the mentioned pathology department.

After routine processing and paraffin embedding, 5 μ m thick sections were cut. The tissue sections were then dewaxed and rehydrated before blocking the endogenous biotin with a blocking kit (Vector Labconsult, Brussels, Belgium), followed by the blockage of non-specific epitopes with 1% BSA in potassium PBS (KPBS, for 1L: 0.2 g KCl, 0.2 g KH_2PO_4 , 2.31 g $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 8 g NaCl, pH 7.4). To assess the binding of biotinylated peptides, they were incubated (overnight, 4°C) with tissue sections at a concentration of 20 μ M diluted in KPBS. The bound peptides were revealed by incubation (1h, RT) with 10 μ g/mL of anti-biotin antibody made in goat and with 10 μ g/mL of fluorescein anti-goat IgG made in rabbit (both from Vector Labconsult) both diluted in phosphate buffer (for 1L: 0.305 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.26 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 8.77 g NaCl, pH 7.8), supplemented with 0.05% Tween-20 and 0.5% BSA for the secondary antibody. The tissue sections were finally mounted with Vectashield Mounting Medium containing 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, Vector Labconsult) and were observed with

a Leica DM2000 microscope equipped with a DFC 425C camera and a light source EL 6000 (Leica Microsystems).

To confirm AdipoR1 (skeletal muscle and pancreas) and AdipoR2 (liver) binding, peptides were co-incubated with AdipoR1/R2-specific antibodies on the same tissue sections, which were submitted to the same pretreatment as described above. The slices were co-incubated (overnight, 4°C) with 20 µM of biotinylated peptides and 4 µg/mL of goat anti-AdipoR1 or anti-AdipoR2 IgG (Santa Cruz Biotechnology, Heidelberg, Germany). Next day, the sections were incubated (1h, RT) with 10 µg/mL of mouse anti-biotin antibody (Vector Labconsult) diluted in phosphate buffer pH 7.8, followed (1h, RT) by a coincubation with 20 µg/mL of hoarse anti-mouse IgG conjugated to Texas Red and 10 µg/mL of rabbit anti-goat IgG conjugated to fluorescein (both from Vector Labconsult) both diluted in phosphate buffer supplemented with 0.05% Tween-20 and 0.5% BSA. The samples were mounted and observed as explained above.

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2.2. Fluorescent colocalization of peptides with insulin on human pancreas

After dewaxing and rehydration, the human pancreas sections were treated with 10 mM sodium citrate (pH 6.0; 0.05% Tween-20) to unmask the epitopes. The endogenous biotin and non-specific epitopes were blocked as described above, followed by overnight coincubation at 4°C with 20 µM of biotinylated peptides and 2 µg/mL of mouse anti-human insulin IgG (Abcam, Cambridge, GB). Then, the sections were incubated (1h, RT) with 10 µg/mL of goat anti-biotin antibody (Vector Labconsult) diluted in phosphate buffer pH 7.8, followed (1h, RT) by a coincubation with 20 µg/mL of hoarse anti-mouse IgG conjugated to Texas Red and 20 µg/mL of rabbit anti-goat IgG conjugated to fluorescein (both from Vector Labconsult) both diluted in phosphate buffer pH 7.8 completed with 0.05% Tween-20 and 0.5% BSA. The samples were mounted and observed as explained above.

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2.3. Fluorescent colocalization of AdipoR1/R2 or of peptides with slow myosin in skeletal muscle

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Slow myosin was colocalized with AdipoR1/R2 on histologic sections from skeletal muscle of NMRI mice. The tissue sections were pretreated as described at point 2.2 (excluding the biotin blocking) and then they were co-incubated (overnight, 4°C) with 20 µg/mL of mouse anti-slow myosin IgG (Abcam) and 4 µg/mL of goat anti-AdipoR1 or anti-AdipoR2 IgG (both from Santa Cruz Biotechnology) diluted in KPBS. Next day, the sections were co-incubated (1h, RT)

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with 20 µg/mL of Texas Red hoarse anti-mouse IgG and 20 µg/mL of fluorescein rabbit anti goat IgG (both from Vector Labconsult) diluted in phosphate buffer completed with 0.05% Tween-20 and 0.5% BSA.

To colocalize biotinylated peptides with slow myosin, the tissue sections were pre-treated identically (but biotin blocking was included) to those used for AdipoR1/R2 colocalization with slow myosin. Then, tissue sections were co-incubated (overnight, 4°C) with 20 µM of biotinylated peptides and 20 µg/mL of mouse anti-slow myosin IgG (Abcam) diluted in phosphate buffer pH 7.4 containing 0.1% Tween-20. Next day, the sections were incubated (1h, RT) with 5 µg/mL of goat anti-biotin antibody (Vector Labconsult) diluted in phosphate buffer pH 7.8, followed (1h, RT) by co-incubation (1h, RT) with 20 µg/mL of Texas Red hoarse anti-mouse IgG and 20 µg/mL of fluorescein rabbit anti goat IgG (both from Vector Labconsult) diluted in phosphate buffer completed with 0.05% Tween-20 and 0.5% BSA. The samples were mounted and observed as explained above.

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2.4. Evaluation of peptides on cell models

2.4.1. Cell lines, culture conditions and induction

The cellular effects of selected peptides were studied on HepaRG human hepatocytes (Life Technologies, Gent, Belgium) and C2C12 myoblasts (kindly offered by Dr. Frédérique Coppée from the Molecular Biology Department of UMONS).

HepaRG cells were cultivated (37 °C, 5% CO₂) in Williams' E medium supplemented with 10% Fetal Bovine Serum (FBS), 1% glutaMAX and 13% Thaw, Plate & General Purpose Supplement (TPGPS). For cellular tests, HepaRG cells were grown in this culture medium for 96 hours (renewed after 48h). Then, the cells were grown for 72 hours in the culture medium comprising the Maintenance/Metabolism Medium Supplement at the place of TPGPS, according to the manufacturer instructions. Finally, the cells were incubated in the culture medium comprising the Induction Medium Supplement (all from Life Technologies) instead of TPGPS, in addition to the tested compounds as described below.

C2C12 cells were grown (37 °C, 5% CO₂) in DMEM medium containing high glucose concentration (4.5 g/L) and glutamine, and supplemented with 10% FBS and 1% penicillin/streptomycin (all from Life Technologies). For various experiments, C2C12 cells were grown in this culture medium for 5 days, when differentiation was induced by three days incubation in the same medium comprising 2% horse serum instead of FBS. After 72h, 33% of the current differentiation

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medium was removed and completed with an equal amount of fresh differentiation medium comprising various compounds as described below.

Once HepaRG and C2C12 cells were prepared for experimental procedures, they were induced for 15, 60 and 135 min in the final culture medium comprising a supplement of 14 mM glucose (25 mM total glucose for HepaRG and 39 mM total glucose for C2C12) or a solution of free fatty acids and cholesterol (FFAC, Chemically Defined Lipid Concentrate, Life Technologies) or both. The FFAC solution was diluted 100 times in the induction medium to obtain 1.18 mM saturated FFA, 1.53 mM unsaturated FFA and 5.69 mM cholesterol. Excepting the control samples, the induction medium furthermore included 40 μ M of one of the assessed non-biotinylated peptides (P16, P17, P18) or a commercial AdipoR agonist (AgoAdipoR; compound 112254, Santa Cruz Biotechnology) at a concentration of 2 μ M.

For immunofluorescence studies, the cells were seeded (2×10^5 HepaRG cells/200 μ L; 8×10^4 C2C12 cells/200 μ L) on microscope coverslips pre-coated with 200 μ L of 200 μ g collagen/mL (type I collagen from rat tail, Sigma-Aldrich, Diegem, Belgium) placed in 6-well culture plates (CellStarTM, Greiner BioOne). For the measurement of phosphorylated AMPK α [pT172] (Life Technologies), the cells were seeded (10^5 HepaRG cells/120 μ L/well; 5×10^4 C2C12 cells/120 μ L/well) in 96-well culture plates (Greiner BioOne). Then, the cells were grown and induced as described above.

All the experiments were performed in triplicates or quadruplicates. For immunofluorescence studies, 6-10 microscopic fields were photographed on each slide using a Leica DM2000 microscope. The fluorescent labeling observed on microphotographs was semiquantitatively analyzed using the ImageJ software (National Institutes of Health, USA).

2.4.2. Detection of AdipoR1 and AdipoR2 by immunofluorescence

After cell culture and induction as described at point 2.4.1, the cell samples were fixed with 4% formaline (15 min, RT) followed by rinsing with 1 mL/well of KPBS. Then, they were blocked (1h, RT) with 1% BSA in KPBS before incubation (overnight, 4°C) with 4 μ g/mL of goat anti-AdipoR1 (for C2C12) or anti-AdipoR2 (HepaRG) IgG (both from Santa Cruz Biotechnology). The bound primary antibodies were detected (1h, RT) with 10 μ g/mL of fluorescein anti-goat IgG made in rabbit prepared in phosphate buffer pH 7.8 containing 0.5% BSA. The cell samples were mounted with Vectashield Mounting Medium with DAPI before observing at microscope.

2.4.3. Quantification of phosphorylated AMPK α [pT172]

The AMPK α phosphorylated on Thr172 [pT172] (AMPK α -pT172) was quantified on cell samples using a sandwich ELISA kit (Life Technologies), comprising two anti-AMPK α -pT172 antibodies, one for the antigen capture and the second one for detection. The cells were cultured and induced as described at point 2.4.1, and then total proteins were extracted with an extraction buffer (Life Technologies) containing a protease inhibitor cocktail (Sigma-Aldrich) and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), according to the manufacturer instructions. For protein extraction, the buffer was incubated (50 μ L/well, 30 min, 4°C) with cells, the culture plate being agitated vigorously every 10 min. The cells were furthermore removed from each well surface by vigorous individual pipetting. Finally, the plate was centrifuged for 5 min at 3000 rpm and 4°C, and the supernatant was transferred in Eppendorf tubes to be again centrifuged for 10 min at 13.000 rpm and 4°C. The cell lysates were stored at -80°C before their use for AMPK α -pT172 quantification, which was performed according to the supplier's instructions, using a calibration curve to obtain AMPK α -pT172 concentration in U/mL. The results were normalized to the protein concentration of each sample, as estimated with the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific).

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2.4.4. Immunofluorescent detection of glucokinase and succinate dehydrogenase

Glucokinase (GK) and succinate dehydrogenase (SDHA) were detected concomitantly by immunofluorescence on cell samples cultured and induced as explained above (point 2.4.1), with the sole difference that cells were induced for only 60 min. Subsequently, the cell samples were fixed (-20°C, 10 min) with 2 mL/well of 100% methanol, followed by rinsing with 1 mL/well of KPBS and blockage/permeabilization (1h, RT) with 1% BSA and 0.3% Triton-X100 in KPBS. GK was detected with 5 μ g/mL of rabbit anti-GK antibody, while SDHA was labeled with 5 μ g/mL of mouse anti-SDHA antibody (both from Abcam), both diluted in KPBS. After overnight incubation at 4°C, the bound primary antibodies were detected by co-incubation of cells (1h, RT) with 20 μ g/mL of Texas red conjugated anti-mouse IgG developed in horse and 20 μ g/mL of fluorescein conjugated anti-rabbit IgG developed in goat (both from Vector Labconsult) diluted in phosphate buffer pH 7.8 containing 0.5% BSA. Finally, the cell samples were mounted and observed at microscope as described above.

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2.4.5. Fluorescent colocalization of AdipoR1 with lysosomes and caveolae on C2C12 cells

AdipoR1 was colocalized with lysosomes and caveolae on differentiated C2C12 cells cultured and induced as described at point 2.4.1, excepting the
5 induction time that was limited to 60 min. The cells were fixed and blocked as explained at point 2.4.5, and then they were co-incubated (overnight, 4°C) with 4µg/mL of goat anti-AdipoR1 IgG and 4 µg/mL of either rabbit anti-LAMP-1 IgG or rabbit anti-caveolin-1 IgG, prepared in phosphate buffer pH 7.8 containing 0.5% BSA. Next day, the bound primary antibodies were detected by co-incubation (1h,
10 RT) with 10 µg/mL of Dylight 594 conjugated horse anti-goat IgG and 20 µg/mL of Dylight 488 conjugated horse anti-rabbit IgG (both from Vector Labconsult). The cell samples were finally mounted and observed at microscope as described above.

3. In vivo evaluation of the agonist property of peptide P17

15 The *in vivo* experiments were performed on 4 groups composed of 4 db/db mice each (BKS(D)-Leprdb/JOrIRj, Janvier Labs, St. Berthevin, France), which received the following treatment and diet (provided *ad libitum*): (a) mice aged of 6 weeks at the beginning of treatment, injected with peptide P17 and fed on a Western high-fat diet containing 60 kcal % fat (D12492, Rodent Diet with 60 kcal % fat,
20 Research Diets Inc., New Brunswick, USA); (b) mice aged of 6 weeks at the beginning of treatment, injected with PBS and fed on a Western high-fat diet containing 60 kcal % fat; (c) mice aged of 7 weeks at the beginning of treatment, injected with peptide P17 and fed on a standard chow diet containing 10 kcal % fat (D12450, Rodent Diet, no sucrose, Research Diets Inc.); (d) mice aged of 7 weeks
25 at the beginning of treatment, injected with PBS and fed on a standard chow diet containing 10 kcal % fat. P17 was injected i.p. each evening at a dose of 40 µmol/kg b.w. (60 µL/30 g of b.w.) for a duration of 4 days. The control mice received PBS that was injected at the same volume and manner as P17. A supplementary group of 4 healthy NMRI mice (Janvier Labs) was used as a witness group of subjects that
30 received a standard chow diet and no substance administration.

The body weight and glycemia of mice were evaluated regularly during the experimental period. The mice were euthanized (injection of 500 mg/kg b.w. of Nembutal and of 0.05 mg/kg b.w. of buprenorphine) the fifth day after the beginning of treatment, when the blood plasma and several tissues and organs (liver, skeletal
35 muscle, pancreas) were collected for supplemental analysis. The triglycerides and adiponectin were measured in blood plasma, while specific biomarkers of adiponectin signaling pathway were analyzed on histologic samples of the collected

tissues and organs (fixed in 4% paraformaldehyde and paraffin embedded). The following biomarkers were studied: AdipoR1 (skeletal muscle), AdipoR2 (liver), AMPK α -pT172 (skeletal muscle and liver) and PPAR α -pS12 (liver). Insulin and glucagon were detected in pancreas samples and were colocalized with activated caspase-3 as a biomarker of apoptosis, aiming to evaluate the reported beta cell depletion in db/db mice. The presence of apoptotic cells was also investigated in liver samples, where the characteristic hepatic steatosis in db/db mice could increase the hepatocyte cell death by apoptosis. Finally, the general tissue integrity was observed in liver by the Masson's trichrome staining.

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3.1. Measurement of blood plasma biomarkers

Blood glycemia was measured in a drop of blood taken from the caudal vein, using the OneTouch® Verio Blood Glucose Meter (Johnson & Johnson Company).

For the measurement of plasma triglycerides and adiponectin, the blood was collected on heparin from mice after euthanasia and blood plasma was separated by centrifugation (30 min, 7000 rpm). The plasma triglycerides were quantified using the triglyceride dosing kit from BioAssay Systems (Gentaur BVBA, Kampenhout, Belgium). Mouse adiponectin was measured in plasma using an ELISA kit from Invitrogen (Life Technologies). Both biomarkers were quantified according to the manufacturers' protocols.

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3.2. Immunofluorescent detection of AdipoR1/R2, AMPK α -pT172, PPAR α -pS12, insulin, glucagon and of activated caspase-3

The tissue sections (liver, skeletal muscle and pancreas) of 5 μ m were dewaxed and rehydrated before blocking (1h, RT) the non-specific epitopes with Protein-Free (TBS) Blocking Buffer (Pierce, Thermo Fisher Scientific). In the case of AdipoR1/R2 detection, the non-specific epitopes were blocked (1h, RT) with 1% BSA in KPBS.

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AdipoR1 and AdipoR2 were detected with 4 μ g/mL of goat anti-AdipoR1 or anti-AdipoR2 IgG (Santa Cruz Biotechnology) incubated overnight at 4°C. Next day, the sections were incubated (1h, RT) with 10 μ g/mL of horse anti-goat IgG conjugated to fluorescein (Vector Labconsult) diluted in phosphate buffer supplemented with 0.05% Tween-20 and 0.5% BSA.

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To stain AMPK α -pT172, tissue sections were incubated (overnight, 4°C) with 2 μ g/mL of rabbit anti- AMPK α -pT172 antibody (Santa Cruz Biotechnology), followed

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by 15 µg/mL of Dylight 488 conjugated anti-rabbit IgG developed in horse diluted in phosphate buffer comprising 0.05% Tween-20 and 0.5% BSA.

PPAR α -pS12 was observed by incubating (overnight, 4°C) tissue sections with 5 µg/mL of rabbit anti-PPAR α -pS12 (Thermo Fisher Scientific), followed by 0.02
5 µg/mL of horse anti-rabbit IgG coupled to fluorescein (Vector Labconsult) diluted in phosphate buffer containing 0.5% BSA.

Activated caspase-3 was stained on liver sections by incubation (overnight, 4°C) with 5 µg/mL of rabbit anti-activated caspase-3 antibody (Thermo Fisher Scientific). Apoptotic livers from Balb/c mice injected i.v. with 1 mg/kg b.w. of anti-
10 Fas antibody (clone Jo2, isotype λ 2, BD Biosciences Pharmingen, Erembodegen, Belgium) were used as positive controls. Then, sections were incubated (1h, RT) with 15 µg/mL of Dylight 488 conjugated anti-rabbit IgG made in horse, diluted phosphate buffer containing 0.5% BSA.

On pancreas sections, activated caspase-3 was co-localized with insulin or
15 glucagon by co-incubation (overnight, 4°C) with 5 µg/mL of rabbit anti-activated caspase-3 antibody (Thermo Fisher Scientific) and 0.2 µg/mL of anti-insulin antibody (clone E2E3, Abcam) or with 2 µg/mL of anti-glucagon antibody (clone K79bB10, Sigma-Aldrich), both produced in mouse.

Finally, all tissue sections were mounted with Vectashield Mounting Medium
20 with DAPI before observing at microscope.

3.3. Evaluation of liver morphology by Masson's Trichrome staining

The liver morphology and integrity were evaluated by staining tissue sections with Masson's Trichrome stain (Accustain® kit, Sigma-Aldrich) performed according
25 to the manufacturer's protocol. Briefly, the nuclei were stained in black with Weigert's iron hematoxylin, while cytoplasm was stained in red with Beibrich scarlet-acid fuchsine. The collagen is stained in blue with aniline blue after treating sections with phosphotungstic and phosphomolybdic acid. The tissue sections were then rinsed in acetic acid and distilled water and mounted in a permanent medium after
30 dehydration.

3.4. Statistical analysis

The results are expressed as means $\pm$ standard deviation (SD). Statistical differences between experimental groups were calculated by one-way ANOVA using
35 the SigmaPlot 11.0 software. Holm-Sidak and Bonferroni corrections were applied

for the groups with uneven variance. A p-value inferior to 0.05 was considered as significant.

Results and discussion

1. Targeting of adiponectin receptors by phage display

5 The homologous amino acid sequence (AdipoR-12C) identified within the C-terminal domain of AdipoRs are ³⁵¹HFYGVSNLQEFR³⁶¹ in AdipoR1 and ³⁶²HFHGVSNLQEFR³⁷³ in AdipoR2, respectively, the only difference between them being thus at the third amino acid position. According to earlier publications, the C-terminal extracellular region of AdipoR1 started at L358, whereas that in AdipoR2
10 had L294 as the starting residue. According to the crystal structures of AdipoR published in 2015, AdipoR-12C belonged to the end of the 7th transmembrane domain (TMD7) (Figure 1A-D). However, the four last residues (QEFR) of AdipoR-12C were assigned by the UniProt protein data bank to the C-terminal extracellular region of AdipoR. Recent studies have moreover suggested that the C-terminal turns
15 of TMD7 may be involved in the recognition of adiponectin by AdipoR1. As our experimental studies reported in this work will reveal, the targeting of AdipoR-12C (HFYGVSNLQEFR) with phage display derived peptides allowed us to modulate different metabolic pathways on cell models and *in vivo*.

 As revealed *in silico* by BLAST (The Basic Local Alignment Search Tool)
20 analysis, the three other extracellular loops of AdipoRs present either a lower degree of homology or a sequence length shorter than 12 amino acid residues. Regarding the homology, we preferred to simultaneously target AdipoR1 and AdipoR2 with the goal to concomitantly modulate both glucose (mainly regulated by AdipoR1) and lipid (mainly regulated by AdipoR2) metabolism, simulating in this way the
25 physiological activity of adiponectin. Concerning the length of the targeted protein fragment, we have observed during our previous studies that fragments shorter than 12 amino acid residues lead to the selection of peptide candidates exposed in duplicate on the phage capsid and are probably meant to equilibrate the molecular interaction.

30 With these concepts and goals in mind, AdipoR-12C has been screened by phage display using a linear 12-mer random peptide library. During the three rounds of panning, the affinity for AdipoR-12C of the phage pools has increased from 1.79 to 2.53 times over that for BSA (Figure 1E), demonstrating an increased specificity. The specific binding to AdipoR1 was even better, the ratio over BSA increasing from
35 0.95 to 7.08 (the 2nd round) and 6.58 (the 3rd round) (Figure 1F). Based on these data, 50 clones were isolated from the 3rd round of panning and their binding to

AdipoR-12C was assessed (Figure 1G). Among them, 20 clones presented a ratio AdipoR-12C/BSA superior to the mean (>1.6) and were selected for supplemental characterization.

5 2. Amino acid sequence and in vitro characterization of the selected peptide clones

The 20 lead phage clones express 12 different peptides (Table 1). They are generally expressed by one clone each, excepting peptides 3, 9 and 10 that are associated to 3 or 4 clones. Several amino acids (G, A, P, K, R, H, S, T, W) are more frequent (Figure 2A) and some of them form consensus motifs (i.e., SWR, GS, RTS) repeated in different clones (Table 1). These amino acids are either basic (K, R, H), uncharged polar (S, T) or hydrophobic (G, A, P, W), which is quite similar to the amino acid composition of AdipoR-12C (16.67% basic, 41.67% hydrophobic, 33.33% uncharged polar) (Table 2), meaning that their interaction could occur via hydrogen bonds, hydrophobic attraction and saline bridges. The presence of hydrophobic amino acids could also promote a closer attraction to the cell membrane.

Table 1. Amino acid sequence of the selected peptides. The consensus amino acid motifs are underlined

SEQ ID n°	Expressing clones	Amino acid sequence
1	Clone 2	VH <u>W</u> DFRQWWQPS
2	Clone 3	AHAHTNWT <u>S</u> WWE
3	Clone 4, 6, 32	DLVSWAG <u>S</u> GKKH
4	Clone 5 (P16)	ADWYHWR <u>S</u> HSSS
5	Clone 8 (P17)	IPNYSMQ <u>S</u> REYR
6	Clone 11	HYR <u>P</u> FTQEHRVT
7	Clone 13	HSFKGWD <u>W</u> PRLR
8	Clone 21	GWKS <u>H</u> EPKGHGS
9	Clones 22, 28, 33, 41, 42	HSFKWLD <u>S</u> PRLR
10	Clone 26, 37, 45	GAYTSWRT <u>S</u> TNA
11	Clone 29	EHLHASW <u>N</u> FSSG
12	Clone 43 (P18)	YDVPNK <u>S</u> WRTSW

Table 2. Theoretical biochemical parameters of peptides P16, P17 and P18, compared to AdipoR1-12C and AdipoR2-12C

Biochemical parameter	P16	P17	P18	AdipoR1-12C	AdipoR2-12C
pI	6.96	8.59	8.59	6.75	6.92
LogP	-3.41	-4.65	-0.73	2.57	0.93
LogD (pH 7.4)	-11.46	-11.71	-9.88	-6.13	-6.13
A.I.	8.33	32.5	24.17	56.67	56.67
Half-life	4.4 h	20 h	2.8 h	3.5 h	3.5 h
Residues (%)					
Acid	8.3	8.3	8.3	8.3	8.3
Basic	25	16.7	16.6	16.7	25
Nonpolar	25	41.6	41.8	41.7	41.7
Polar non-ionizable	41.7	33.4	33.3	33.3	25

pI: isoelectric point; LogP: partition coefficient; LogD: distribution coefficient; A.I.: aliphatic index: the relative volume occupied by aliphatic side chains; half-life was theoretically estimated in mammalian reticulocytes *in vitro* according to the N-end rule.

- 5 ExpPASy proteomics server was used to estimate pI, A.I. and half-life.
ACD/ChemSketch 2.0 software was used to calculate LogP.
MarvinSketch 5.11.5. software (<http://www.chemaxon.com>) was used to calculate LogD.

- 10 The affinity for AdipoR1 of the identified peptide clones was evaluated and compared to that for BSA, which was negligible. Based on the K_d values, three clones were selected for additional characterization, namely 5, 8 and 43 (Figure 2B). They were encoded as P16 (ADWYHWRSHSSS), P17 (IPNYSMQSREYR) and P18 (YDVPNKSWRTSW). The three-dimensional structure and spatial conformation of
15 these peptides is compared to AdipoR1-12C and AdipoR2-12C in Figure 3.

- The exposure to the solvent of hydrophobic residues like Trp, Tyr, Phe, which are mainly located at the peptide extremities, are remarkable both in the three hit peptides (P16, P17 and P18) and in AdipoR-12C. This property may be advantageous for the interaction of our peptides with AdipoR in the close proximity
20 of cell membrane. The proportion of residues with different chemical functions is almost identical in P17, P18 and AdipoR1-12C and similar to AdipoR2-12C (Table 2). All of them are rather hydrophilic, with a slightly higher tendency to hydrophobicity for AdipoR1, as suggested by their LogP and LogD values. At the same time, the A.I. and proportion of nonpolar residues suggest that P17 and P18
25 present a more hydrophobic character than P16, being closer to AdipoR-12C and

predicting their ability to interact with both the target and cell membrane. Among all the analyzed peptides, P17 is characterized by the best theoretical half-life, namely 20 hours, which highlights it as a promising pharmacological candidate for *in vivo* applications.

- 5 The alignment of the three candidate peptides with relevant proteins was analyzed using the BLAST tool of the NCBI proteomics server, showing interesting homologies with proteins involved in signal transduction of various growth factors and hormones, including insulin, metabolism, cell proliferation and differentiation, and DNA repair (Tables 3, 4, 5).

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Table 3. Sequence alignment of peptide P16 with relevant human protein sequences as identified with BLAST of the NCBI proteomics server using the UniProtKB/Swiss-Prot database

Protein	SwissProt ID	Peptide alignment	Function
Probable phospholipid-transporting ATPase VD	Q9P241	⁷ W--YHWR ¹³	Role in membrane translocation of phospholipids
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit beta isoform	Q15173	⁴³ RSHSSS ⁴⁸	Involved in dephosphorylation of protein kinase AKT1 at Thr308 and Ser373
Phosphatidylglycerophosphatase and protein-tyrosine phosphatase 1	Q8WUK0	³⁶ DWYH ³⁹	Role in dephosphorylation of phosphatidylglycerophosphate, ATP production, probably involved in insulin secretion by pancreatic beta cells
1,4-alpha-glucan-branching enzyme	Q04446	¹⁷⁴ DW-HW ¹⁷⁸	Role in intracellular accumulation of glycogen

- 15 For instance, P16 presents homologies with proteins involved in membrane translocation of phospholipids, protein and lipid phosphatases involved in insulin signaling and secretion, or an enzyme playing a role in glycogen accumulation (Table 3). The analysis of peptide sequence of P17 reveals homologies with proteins involved in insulin sensitivity, cell proliferation, the defense against cell stress and
- 20 control of protein folding. Regarding P18, this one shows sequence homologies with several proteins playing a role in cell cycle, DNA repair, intracellular trafficking of proteins and signal transduction.

Table 4. Sequence alignment of peptide P17 with relevant human protein sequences as identified with BLAST of the NCBI proteomics server using the UniProtKB/Swiss-Prot database

Protein	SwissProt ID	Peptide alignment	Function
Syntaxin-4	Q12846	¹⁴³ S ¹⁴⁹ MQS-EYR	Involved in the docking of intracellular vesicles to the plasma membrane, including that of intracellular GLUT4-containing vesicles in adipocytes
Insulin-like growth factor-binding protein 3	P17936	²⁸⁶ YSMQS ²⁹⁰	Role in the regulation of IGFs effects on cell proliferation
Cytoplasmic tyrosine-protein kinase BMX	P51813	²²⁸ PN--MQ--- RE ²³⁸	Involved in signal transduction of growth factor receptors, cytokine receptors or G-protein coupled receptors and in cell differentiation induced by IL-6, against extracellular stress
Coenzyme Q-binding protein COQ10 homolog A, mitochondrial	Q96MF6	⁹⁵ YSMQ-----S- -REYR ¹¹¹	Involved in the function of coenzyme Q in the mitochondrial respiratory chain
Torsin-1A	O14656	²⁸⁵ MQSR-Y ²⁹⁰	Role in the control of protein folding

5 **Table 5.** Sequence alignment of peptide P18 with relevant human protein sequences as identified with BLAST of the NCBI proteomics server using the UniProtKB/Swiss-Prot database

Protein	SwissProt ID	Peptide alignment	Function
Serine/threonine-protein kinase tousel-like 1	Q69YN2	⁴⁹⁰ NKSWR ⁴⁹⁴	Role in cell cycle, the repair of DNA double-stranded breaks,

			intracellular trafficking of proteins and signal transduction
Lanosterol 14-alpha demethylase	Q16850	¹⁴⁵ YDVPN ¹⁴⁹	Role in steroid biosynthesis
Glucocorticoid receptor	P04150	⁵⁴⁸ YD--VP+-- WR ⁵⁵⁸	Role in cell response to glucocorticoids, transcription factor, cell proliferation and differentiation, inflammatory response, may play a negative role in adipogenesis
Serine/threonine-protein phosphatase CPPED1	Q9BRF8.3	⁹⁶ P-K-WRT ¹⁰²	Role in AKT protein dephosphorylation, may play an inhibitory role in glucose uptake by adipocytes
Glycogen debranching enzyme	P35573.3	⁹²³ YD+PN-- W ⁹²⁸	Involved in glycogen degradation
Acyl-coenzyme A thioesterase THEM4	Q5T1C6.1	⁴⁸ VPN-SW ⁵³	Role in fatty acid metabolism, involved in insulin receptor signaling pathway

3. In vitro characterization of the candidate hit peptides

3.1. Validation of peptide binding to AdipoR1 and AdipoR2 in muscle, liver and pancreas

- 5 AdipoR1 is known to be mainly expressed in skeletal muscle, where it activates the signaling pathway of AMPK, while AdipoR2 is predominantly expressed in liver, where it activates the pathway of PPAR α . The AMPK activation by phosphorylation (AMPK-p) increases the fatty acid β -oxidation and glucose uptake via the membrane translocation of glucose transporter GLUT4 (in muscle) or GLUT2
- 10 (in liver) and inhibits gluconeogenesis (in liver). PPAR α plays major roles in lipid and glucose metabolism and exerts anti-inflammatory effects. In pancreatic beta cells, the level of AdipoR1 and AdipoR2 expression is comparable to that in liver and greater than in muscle, but AdipoR1 isoform is predominant at least in mouse islets. However, it seems that AMPK pathway is not activated in pancreatic beta cells,

where Akt protein kinase and extracellular signal-regulated kinase (ERK) are involved in adiponectin signaling to protect against apoptosis and stimulate insulin expression and secretion.

During the first step of validation, the binding of candidate hit peptides P16, P17 and P18 to AdipoR1 in mouse skeletal muscle and human pancreas (Figures 4A and 5) and to AdipoR2 in mouse liver (Figure 4B) was evaluated by immunofluorescence. The colocalization of peptides with AdipoR1 and AdipoR2 has been quantified by estimating the Manders' Colocalization Coefficients (MCC) M1 (overlap of red channel over the green channel) and M2 (overlap of green channel over red channel) using the ImageJ software.

Adiponectin receptors were observed at the level of cell membranes, but also in the cytoplasm, mainly in the skeletal muscle. According to literature, AdipoR1 and AdipoR2 can be detected at the level of cellular organelles when cells are permeabilized, which is the case of our tissue samples. Intracellularly, they were detected in the endoplasmic reticulum (ER), where they dimerize and interact with ER protein 46 (ERp46) that modulates adiponectin signaling, in caveolae, where AdipoR1 interacts with adaptor protein containing pleckstrin homology domain (APPL1) and adenylate cyclase within a signaling complex requiring caveolin-3, and in clathrin-coated endosomes that regulate AdipoR recycling and degradation.

Regarding the colocalization of peptides with AdipoR1 in skeletal muscle and AdipoR2 in liver, the MCC results suggest that P18 is highly specific to AdipoR1 in muscle (Figure 4A), while P16 and P17 are highly specific to AdipoR2 in liver (Figure 4B). In the case of muscle, P16 and P17 were predominantly observed at the level of cell membrane, although AdipoR1 was detected in the cytoplasm too, which is at the origin of lower MCC values.

At the level of human pancreas, the best colocalization with AdipoR1 is shown by the MCC values of P17, although all three peptides were specifically detected in the pancreatic islets (Figure 5A) considering that exocrine pancreas was not stained. Aiming to verify the peptides' binding to beta cells, they were colocalized with insulin (Figure 5B). The MCC data suggest that peptides either bind to other cell types in pancreatic islets in addition to beta cells, or they bind to AdipoR2 in addition to AdipoR1.

During our studies performed on muscle, we have observed that some muscle fibers presented a lesser staining for AdipoR1 than others, and we have hypothesized that its expression could depend on the fiber type, namely slow-twitch (type I) and fast-twitch (type II) fibers. To corroborate this hypothesis, slow-twitch fibers were detected by staining the slow myosin, which was co-localized with AdipoR1 or AdipoR2 (Figure 6A). Our results reveal that AdipoR1 is weakly

expressed by slow-twitch fibers characterized by a highly oxidative metabolism. Conversely, AdipoR1 is better expressed by fast-twitch fibers that present a high glycolytic capacity, confirming the specialized literature. Concerning AdipoR2, this one seems to be more homogenously expressed by both fiber types, presenting however a lower expression in about a half of slow-twitch fibers. We have then evaluated the binding of our three candidate peptides to these two fiber types (Figure 6B) and observed that P16 presents a better binding to fast-twitch fibers (and probably to AdipoR1), whereas P17 and P18 distribute to both fiber types, probably interacting with both AdipoR1 and AdipoR2.

Altogether, these results show that adiponectin receptors are predominantly located at plasma membrane, but also in the intracellular compartment. At least in mouse skeletal muscle, AdipoR1 is mainly expressed by fast-twitch type II fibers, whereas AdipoR2 is expressed by both fiber types, although its distribution in slow-twitch type I fibers is more heterogenous. Among the three candidate peptides, P17 and P18 seem to bind both AdipoR1 and AdipoR2 in type I and type II muscle fibers, while P16 is more specific to AdipoR1 in fast-twitch type II fibers. P16 and P17 bind with high efficacy to AdipoR2 in liver and are able to concentrate in human pancreatic islets, where they could probably recognize both AdipoR1 and AdipoR2.

3.2. Cellular activity of peptides

3.2.1. Expression of adiponectin receptors in HepaRG cells and differentiated C2C12 cells as a function of culture medium composition

It has been shown that AdipoR1 (but not AdipoR2) expression is significantly enhanced after 48 hours of starvation, while a high-fat meal diminishes AdipoR1 expression, AdipoR2 being less sensitive to dietary fat. At the gene level, AdipoR1 (but not AdipoR2) promoter activity is repressed (in C2C12 myoblasts) by insulin via the PI3K/Foxo1 pathway. In L6 rat skeletal muscle cells, AdipoR1 expression was decreased by hyperinsulinemia and hyperglycemia, whereas AdipoR2 expression was stimulated by hyperinsulinemia. In addition, AdipoR1 promoter harbors a responsive element recognized by the ER stress-inducible activating transcription factor 3 (ATF3) able to downregulate AdipoR1 expression in C2C12 and HepG2 cells, which may be responsible of impaired AdipoR1 signaling in obese and diabetic patients.

Aiming to evaluate the effect of peptides on AdipoRs signaling in hepatocytes (HepaRG cell line) and skeletal muscle cells (differentiated C2C12 cells) under high-glucose and high-fat culture conditions, we have first investigated AdipoR1 and

AdipoR2 expression induced by high glucose (25 mM for HepaRG; 39 mM for C2C12) and lipid (free fatty acids and cholesterol, FFAC) supplements in the culture medium. AdipoRs expression has been semi-quantitatively evaluated by immunofluorescence and monitored after 15, 60 and 135 minutes of induction (Figure 7). In HepaRG cells (Figure 7A and 7C), AdipoR2 expression was significantly inhibited after 60 min of incubation with 25 mM glucose ($p < 0.01$ vs. FFAC at 60 min, and vs. glucose at 15 and 135 min). At the same time point, AdipoR2 expression was amplified in HepaRG cells induced with both glucose and FFAC ($p < 0.01$ vs. glucose at 60 min, and vs. glucose & FFAC at 15 and 135 min). In differentiated C2C12 cells (Figure 7B and 7C), AdipoR1 presented higher levels of expression at all incubation times, but principally at 60 min, when the cells were incubated with both glucose and FFAC ($p < 0.01$ vs. all experimental conditions in C2C12 cells). Glucose also induced significant AdipoR1 expression after 60 min of induction ($p < 0.01$ vs. glucose at 15 and 135 min).

We can thus conclude that high glucose concentration induces AdipoR1 expression in C2C12 cells, but inhibits that of AdipoR2 in HepaRG cells, this effect being observed after 60 min of incubation. When HepaRG and C2C12 cells were incubated with both high glucose concentration and FFAC, AdipoR1 and AdipoR2 were highly expressed after 60 min of incubation. This phenomenon could be involved in postprandial activation of AdipoRs to assist the homeostasis of glucose and lipid metabolism and be explained by the regulation of pre- and posttranslational molecular mechanisms, including those responsible of AdipoRs dimerization and endocytosis.

3.2.2. Modulation of AMPK activity

Subsequent to adiponectin binding to the extracellular region of AdipoRs, their intracellular region interacts with APPL1 adaptor protein, which activates the liver kinase B1 (LKB1) and Ca^{2+} /calmodulin-dependent protein kinase kinase 2 (CaMKK2), responsible of AMPK phosphorylation. AMPK is activated by the AMP binding to its γ subunit, which induces a conformational modification of a subunit harboring the catalytic domain of AMPK. This last one exposes thus its Thr172 residue that is phosphorylated by LKB1, which fully activates AMPK. The same residue is also phosphorylated by CaMKK2, but only when intracellular Ca^{2+} concentration is elevated following the APPL1-induced opening of Ca^{2+} channels during AdipoR1 activation by adiponectin. The two pathways of AMPK activation can operate simultaneously when both AMP and Ca^{2+} concentrations are increased

intracellularly. Once activated, AMPK is involved in the fatty acid oxidation and glucose uptake by peripheral tissues.

The ability of our three candidate peptides to bind AdipoRs and activate AMPK phosphorylation was evaluated on HepaRG and differentiated C2C12 cell lines. The cells were incubated with peptides or with the positive control compound Ago-AdipoR for 15, 60 and 135 minutes in two types of culture media. The first one was supplemented with 14 mM glucose (total glucose concentration in the culture medium was of 25 mM for HepaRG and 39 mM for C2C12) and was complemented or not with a mixture of free fatty acids and cholesterol (FFAC). The second one comprised basal glucose concentration of the culture media (11 mM for HepaRG; 25 mM for C2C12), which was supplemented or not with FFAC. The control group was incubated in the same culture media as the test groups by excluding the peptides and AgoAdipoR. At the end of incubation times, the phosphorylated AMPK (AMPK α -pT172) was quantified by ELISA on protein extracts obtained from cell lysates.

Among the three peptides, P16 presented the weaker effect on AMPK activation and at short incubation times of HepaRG cells, namely at 15 minutes (Figure 8D) and 60 minutes (Figure 8A). This effect was observed in culture media supplemented either with glucose (Figure 8A) or with FFAC (Figure 8D). In C2C12 cells induced with FFAC, P16 even induced a significant inhibition ($p < 0.05$) of AMPK phosphorylation after 15 minutes of incubation.

Peptide P17 induced a significant increase of AMPK α -pT172 concentration in almost all experimental conditions and particularly on HepaRG cells. The higher effect has been observed when HepaRG cells were challenged with both 25 mM glucose and FFAC (Figure 8C). In these cells, AMPK α -pT172 concentration increased three times (~ 123 U/mg protein) as compared to HepaRG cells incubated with 25 mM glucose alone (~ 40 U/mg protein) (Figure 8A) after 60 and 135 minutes of induction with P17. When compared to control HepaRG cells incubated with both 25 mM glucose and FFAC (~ 58 U/mg protein), P17 caused two times increase of AMPK α -pT172 concentration (~ 123 U/mg protein) after 60 and 135 minutes of incubation (Figure 8C); in control cells, FFAC induced a double increase of AMPK α -pT172 concentration (~ 58 U/mg protein) (Figure 8C) in comparison with control cells incubated with 25 mM glucose alone (~ 25 U/mg protein) (Figure 8A). C2C12 cells seem to better respond to high glucose concentration (39 mM) (Figure 8E), but not to FFAC presence (Figures 8G and 8H). The AMPK activation induced by P17 seems to follow AdipoRs expression (Figure 7) in media supplemented with either glucose alone or with glucose and FFAC. No significant effect has been observed on C2C12 cells incubated with 25 mM glucose (basal medium concentration) and FFAC (Figure 8H). These results suggest on the one hand that P17 acts as an agonist of

both AdipoR1 and AdipoR2, and on the other hand that FFAC is an inducer of AMPK phosphorylation in HepaRG cells. In C2C12 cells, only glucose supplement acts as an inducer of AMPK phosphorylation, i.e., ~40 U/mg protein in the presence of 39 mM glucose (Figure 8A) compared to ~27 U/mg protein in the presence of 25 mM glucose (Figure 8F) and ~28 U/mg protein in the presence of 39 mM glucose and FFAC (Figure 8G).

Peptide P18 manifested an optimal effect on AMPK activation in HepaRG cells incubated in culture media supplemented with glucose (Figure 8A), glucose and FFAC (Figure 8C) or FFAC (Figure 8D), demonstrating its agonist activity on AdipoR2 expressing cells. In the case of C2C12 cells, P18 triggered AMPK activation only when cells were challenged for 60 minutes with a supplement of glucose and FFAC (Figure 8G).

In HepaRG cells, the positive control compound, AgoAdipoR, induced a significant increase of AMPK α -pT172 concentration when culture media were supplemented with both glucose and FFAC (Figure 8C) or with FFAC alone (Figure 8D). C2C12 cells were stimulated by AgoAdipoR to activate AMPK by phosphorylation when culture media were supplemented with glucose (Figure 8E) or with glucose and FFAC (Figure 8G).

Taken together, these results reveal that P17 is the most potent activator of AMPK phosphorylation, likely by acting as an agonist of both AdipoR1 and AdipoR2. At the same time, FFAC is an inducer of AMPK activity in HepaRG cells, whereas glucose activates AMPK phosphorylation in C2C12 cells. Our results corroborate other published studies that stipulate an allosteric modulation of AMPK by FFA, which renders AMPK susceptible to be phosphorylated by LKB1. In addition, palmitic acid stimulates glucose uptake through the activation of PI3K/AMPK/ERK1-2 pathways, leading to the cell membrane translocation of glucose transporter GLUT4.

3.2.3. Modulation of succinate dehydrogenase and of glucokinase activity in HepaRG and C2C12 cells

Activated AMPK regulates the lipid metabolism by different mechanisms, such as the activation of peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC1 α , stimulates mitochondrial biogenesis), of carnitine palmitoyltransferase-1 (CPT-1, responsible of FFA β -oxidation) and of PPAR α (stimulates the expression of genes involved in FFA β -oxidation), and the inhibition of 5-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, involved in cholesterol production). Glucose metabolism is also regulated by AMPK at different levels, such as the activation of glucose uptake via the membrane

translocation of glucose transporter 4 (GLUT4) and 1 (GLUT1), the inhibition of glucose efflux via GLUT2, the activation of 6-phosphofructo-2-kinase (PFK2) to stimulate glycolysis, and the inhibition of gluconeogenesis via the downregulation of specific gene expression.

5 Based on the known effects of adiponectin related to AMPK activation, the next step of peptide validation involved the characterization of their ability to modulate two enzymes engaged in cell metabolism, namely glucokinase (GK) and succinate dehydrogenase (SDHA). Being the most potent inducer of AMPK phosphorylation, peptide P17 has been selected as a lead compound among the
10 three candidate peptides. Its putative effects on GK and SDHA expression and eventual activation have been assessed on HepaRG and differentiated C2C12 cells incubated in the same culture conditions as for the study of AMPK modulation. GK and SDHA were simultaneously detected by immunofluorescence on the same cell samples, and the relative ratio of fluorescent labeling (RRFL) was semi-
15 quantitatively evaluated using the ImageJ software (Figure 9).

 In control cells incubated in a culture medium supplemented with glucose or glucose and FFAC, the expression of SDHA was significantly ($p<0.01$) enhanced as compared to control cells cultured in the basal medium (Figure 9C). However, GK was significantly ($p<0.01$) induced only in control HepaRG cells challenged with the
20 glucose supplement. GK catalyzes glucose phosphorylation to obtain glucose-6-phosphate (G6P), which is then used either in glycolysis or in glycogen synthesis depending on glucose availability and insulin levels. Its transcription is stimulated by insulin, carbohydrates and fructose-2,6-bisphosphate (F2,6P₂), which is produced by PFK2, an enzyme that is not affected by ATP concentrations.
25 Considering that glucose supplement has induced both GK and SDHA (a mitochondrial enzyme that participates in Krebs cycle and the respiratory chain) in HepaRG cells, we may assume that glucose was metabolized by both glycolysis and oxidative phosphorylation. In the other control conditions, only SDHA was stimulated either by glucose supplement (in C2C12 cells) or by the complement of
30 glucose and FFAC (in HepaRG and C2C12 cells). These results suggest that glycolysis was already enough elevated in C2C12 cells incubated with glucose supplement to provide the pyruvate for Krebs cycle, whereas FFAC were directly metabolized by oxidative phosphorylation in HepaRG and C2C12 cells. At low glucose concentrations, GK binds to its regulatory protein (GKRP) in hepatocytes and moves
35 to the nucleus, where GK is sequestered and inactivated. High glucose concentration releases GK from GKRP and returns to cytoplasm to participate in glucose metabolism. In our experimental conditions, we could not observe GK in the nucleus

of HepaRG cells, probably because glucose concentration was high enough even in the basal medium.

HepaRG and differentiated C2C12 cells cultured in media supplemented with glucose or glucose and FFAC and induced with peptide P17 have all presented a significantly enhanced labeling of GK and SDHA (compared to control samples), suggesting an activation of these two enzymes induced by P17 (Figure 9C). The only condition where GK was not significantly modified corresponded to C2C12 cells incubated in a culture medium complemented with glucose, probably because GK in these cells is sensitive to much larger glucose concentrations. The presence of FFAC induced a striking activation of GK in both HepaRG and C2C12 cells stimulated with P17, probably because of the permissive effect of FFA on glucose uptake. It has been shown that palmitate stimulates glucose uptake via the activation of PI3K/AMPK/Akt and PI3K/ERK1-2 pathways. In differentiated C2C12 cells, P17 has also induced a marked activation of SDHA independently of FFAC presence.

Altogether, these results corroborate the conclusions drawn above regarding AMPK activation, which revealed that FFAC have a permissive effect on AMPK phosphorylation in HepaRG cells, whereas glucose favors AMPK phosphorylation in C2C12 cells. The effects of peptide P17 on GK and SDHA activity seem also to correlate with its ability to induce AMPK phosphorylation (Figure 8) in HepaRG and C2C12 cells, being probably related to its agonist AdipoR1 and AdipoR2 activity. It is known that AMPK increases the FFA uptake (via the plasma membrane translocation of FFA transporter) and their β -oxidation (increases CPT-1 activity and FFA transport into the mitochondria), induces the expression of proteins involved in oxidative phosphorylation (i.e., PGC1 α) and the activities of mitochondrial enzymes (i.e., SDHA), and activates PFK2 by phosphorylation which produces F2,6P₂, an inducer of GK expression and activity.

3.2.4. Colocalization of AdipoR1 with lysosomes and caveolae in C2C12 cells

Under basal conditions, AdipoR1 and AdipoR2 can self-associate to form homomers or heteromers at the plasma membrane. In response to full-length adiponectin (FLAdipoQ), AdipoRs redistribute to early endosomes within the first 5 minutes after stimulation and disappear 30 minutes later. The globular fragment of adiponectin (GAdipoQ) induces AdipoRs endocytosis after 30 minutes of stimulation. After endocytosis, AdipoRs can return to the membrane or be degraded within lysosomes to down-regulate adiponectin signaling. It has also been shown that AdipoR1 and caveolin-1 associate at the plasma membrane of endothelial cells, this association being critical for adiponectin transmembrane signaling. Under high

glucose/high lipids concentration, AdipoR1 interaction with caveolin-1 is downregulated majorly because of the reduced caveolin-1 expression.

Based on these observations, we hypothesized that peptide P17 may modulate AdipoR1 endocytosis via lysosomes (detected with anti-LAMP-1 antibody) or caveolae (detected with anti-caveolin-1 antibody) in differentiated C2C12 cells incubated for 60 minutes in a culture medium supplemented with glucose or glucose and FFAC. AdipoR1, LAMP-1 and caveolin-1 were observed by immunofluorescence, and the level of immunostaining was semi-quantitatively measured with ImageJ software on the acquired microphotographs. Aiming to evaluate the endocytic pathway followed by AdipoR1 in different experimental conditions, the colocalization of AdipoR1 with LAMP-1 or caveolin-1 was analyzed using the MCC M1 (overlap of red channel over the green channel) and M2 (overlap of green channel over red channel) coefficients, quantified by ImageJ software.

The results obtained for AdipoR1 immunostaining suggest that glucose supplement induced a significant decrease of AdipoR1 expression ($p < 0.01$ vs. basal medium) in control cells. At the same time, caveolin-1 immunodetection was significantly increased ($p < 0.01$ vs. basal medium) by glucose supplement, but not that of LAMP-1 that was similar to the cells incubated in the basal medium (Figure 10 and Figure 11). According to literature, the glucose uptake in skeletal muscle cells depends on caveolin-1 expression, which could contribute to insulin sensitivity. We may therefore presume that caveolin-1 expression in our experimental conditions has been induced under high-glucose concentration to assist the glucose uptake. On the other hand, the M1 coefficient reveals an excellent colocalization of AdipoR1 with caveolin-1 ($M1 = 0.945$), but lower for LAMP-1 ($M1 = 0.747$). Considering that caveolin-1 expression was stimulated by glucose supplement in C2C12 cells and its colocalization with AdipoR1 may assist the signaling cascade of this receptor, we could conclude that lysosomal degradation of AdipoR1 may contribute to its downregulation.

In the same culture conditions (medium supplemented with glucose), P17 induced a significant increase of AdipoR1 expression ($p < 0.01$ vs. control; $p < 0.05$ vs. basal medium) (Figure 10 and Figure 11), which was associated with a significant increase of lysosome immunodetection ($p < 0.05$ vs. control) and a significant decrease of caveolae immunostaining ($p < 0.01$ vs. control). However, both lysosomes and caveolae remained at the level of control cells incubated in the basal medium. The colocalization with LAMP-1 was lower ($M1 = 0.539$) than in control cells challenged with glucose, while that with caveolin-1 was similar ($M1 = 0.893$). Although the colocalization of AdipoR1 with LAMP-1 was weak, the induction of lysosome constitution could be a downregulation mechanism of AdipoR1 signaling

cascade, knowing that P17 induced a significant increase of AMPK phosphorylation (Figure 8E) and of SDHA expression (Figure 9C) in the same culture conditions.

The presence of both glucose and FFAC supplement in the culture medium (Figure 10 and Figure 12) induced a prominent and significant increase of AdipoR1 expression ($p < 0.01$ for P17; $p < 0.05$ for control vs. basal medium). In control cells, this was associated with a significant increase ($p < 0.05$ vs. basal medium) of both lysosome and caveolae content. The relatively good colocalization of AdipoR1 with LAMP-1 ($M1 = 0.612$) and better with caveolin-1 ($M1 = 0.870$) suggests that AdipoR1 signaling cascade could be regulated by the two endocytic pathways to assist both nutrient metabolism and receptor downregulation. In the case of cells stimulated with P17, lysosome content was significantly decreased ($p < 0.01$) as compared with control cells, whereas caveolae were comparable to control cells challenged with glucose and FFAC and to those cultured in basal medium. At the same time, AdipoR1 colocalization with LAMP-1 was weak ($M1 = 0.474$), while that with caveolin-1 was very good ($M1 = 0.774$), suggesting that P17 diminishes the lysosome degradation of AdipoR1. In the same culture conditions, P17 induced a significant activation of AMPK (Figure 8G) and of GK and SDHA expression (Figure 9C).

Globally, these results corroborate the published literature, which reported that hyperglycemia is able to downregulate AdipoR1 expression. In C2C12 cells, high glucose concentration has also upregulated caveolin-1 expression, probably to facilitate the glucose uptake in skeletal muscle cells as described by the specialized literature. The glucose and FFAC supplement induced a striking increase of AdipoR1 expression, which was associated with a concomitant increase of lysosome and caveolae content of C2C12 cells. It is known that FFA stimulate glucose uptake (probably sustained in our case by the high caveolin-1 expression) and AMPK activation, which suggests that our results confirm the published literature. Moreover, the moderate AdipoR1 colocalization with LAMP-1 could mean that high AdipoR1 expression may be sustained by an equilibrated lysosomal degradation.

The cells stimulated with peptide P17 presented an elevated AdipoR1 expression, which was associated with a weak colocalization with lysosomes and a very good colocalization with caveolae. These results could reveal on the one hand that P17 limits the lysosomal degradation of AdipoR1, and thus its downregulation, and on the other hand that it improves the caveolin-1/AdipoR1 signalosome formation. This last event is in agreement with the other cellular events upregulated by P17, namely the AMPK phosphorylation and GK/SDHA expression.

4. In vivo evaluation of the selected peptide P17

It is well known nowadays that adiponectin plays multiple roles in protecting against metabolic syndrome, type 2 diabetes (T2D) and cardiovascular disease (CVD). Adiponectin regulates the food intake and body weight, the lipid and glucose metabolism, has an antiatherogenic activity, it improves the insulin sensitivity, and is inversely correlated with CVD risk factors such as blood pressure, low-density lipoproteins (LDL) and triglycerides. Adiponectin expression and secretion are decreased in subjects with obesity, hypertension, T2D or presenting other characteristics that define the metabolic syndrome. Its expression and secretion seem to be regulated by TNF α , probably via an increased production of IL-6.

Based on the *in vitro* studies presented above, peptide P17 has been selected as a potential agonist candidate of adiponectin receptors that may be able to regulate the metabolic state of obese and diabetic subjects. Aiming to evaluate its agonist ability in pathologic conditions, P17 has been assessed on db/db mice, which develop obesity and hyperglycemia starting by 4-5 weeks of age, but also glycosuria, polyuria, polydipsia, polyphagia and insulin resistance. Therefore, this mouse strain is widely used as a model of T2D and obesity.

4.1. The effects on body weight and blood biomarkers

The body weight of the 5 groups of mice was followed during the experimental period of 4 days (Figure 13), showing that mice treated with P17 have the tendency to stabilize their weight as opposed to db/db mice treated with PBS. The mice in 60 kcal group presented a weight gain of 8.04% after 4 days of P17 treatment, whereas those in 10 kcal % group gained 5.78% to their weight after 4 days of P17 treatment, being similar to the weight evolution of healthy NMRI that presented a weight gain of 6.19% at the end of the experimental period. On the contrary, db/db mice treated with PBS have shown a weight gain of 13.11% in 60 kcal % group and of 11.23% in 10 kcal % group. Our results seem to corroborate the effects produced by intracerebroventricular or systemic adiponectin administration in Lep^{ob/ob} mice, which exhibited a weight loss without an inhibition of food intake. This was explained by an increased lipid oxidation and thermogenesis and was also associated with a reduction in serum glucose and lipid levels.

Accordingly, P17 has also induced a significant decrease of glycemia in 60 kcal group after 66h ($p < 0.01$; -37.9% vs. 24h of treatment) and 90h ($p < 0.05$; -34.92% vs. 24h of treatment) of treatment (Figure 4A). In the 10 kcal % group, P17 induced a significant decrease of glycemia ($p < 0.05$; -24.84% vs. 24h of treatment) after 90h of treatment. We have also observed a significantly lower level

of glycemia in the group of mice treated with PBS and fed on a 60 kcal % diet ($p < 0.01$; -37.20% vs. 24h of treatment), which was evident after 90h of treatment and may be explained by endogenous mechanisms of glycemia regulation. Interestingly, the glycemia in healthy NMRI mice was increased by 33.80% at the end of experimental period, although this increase was not significantly different from the values measured at 24h. This may be explained by an increased food intake just before the measurement of glycemia.

The most evident effect produced by P17 administration was observed in the case of plasma triglyceride concentration (Figure 14B), which was significantly reduced in both experimental groups ($p < 0.05$ in 60 kcal % group; $p < 0.01$ in 10 kcal % group) by about 50% as compared to PBS treated controls, arriving in the range of healthy NMRI mice. This could be explained by an increased lipid oxidation through the AdipoRs binding, followed by the activation of AMPK and PPAR α pathways as explained above. It has also been shown that adiponectin reduces plasma triglycerides by increasing the expression and activity of lipoprotein lipase (LPL) in skeletal muscle, as well as the expression of VLDL receptor (VLDLr), enhancing in this way the VLDL-triglyceride catabolism.

It is also interesting that P17 administration has also induced a weak increase of plasma adiponectin concentration (Figure 14B) probably as a consequence of the body weight stabilization and lipid oxidation as described by the specialized literature [7]. Adiponectin plasma concentration was significantly higher ($p < 0.05$) than in healthy NMRI mice in the group of db/db mice treated with P17 and fed on a 60 kcal % diet.

Taken together, these results suggest that P17 contributes to the body weight stabilization, the decrease of glycemia, the striking reduction of plasma triglyceride concentration and the slight increase of adiponectin secretion.

4.2. The effects on adiponectin signaling pathway

FLAdipoQ and GAdipoQ interact with AdipoR1/R2 and stimulate the AMPK and PPAR α activation, which are involved in the FFA oxidation and glucose uptake by the targeted cells. Consequently, the Thr172 residue of AMPK is phosphorylated by LKB1 and CaMKK2, which fully activate AMPK. PPAR α is a nuclear receptor that is indirectly activated by AMPK via the activation of p38 mitogen-activated protein kinase (p38 MAPK), which in turn phosphorylates several serine residues (S6, S12 and S21) of PPAR α . The tissues that obtain most of their energy from FFA oxidation, such as liver, heart, kidney and skeletal muscle, are characterized by high levels of PPAR α expression.

It has been shown that serum levels of adiponectin are lower in subjects with T2D and obesity, while adiponectin administration decreases the plasma levels of FFA and triglycerides, improving thus the insulin sensitivity. Moreover, the mRNAs of AdipoR1/R2 were found to be significantly decreased in skeletal muscle and adipose tissue of ob/ob mice, whereas they were increased in the liver of insulin resistant obese patients, where the high AdipoRs expression was proposed to contribute as a compensatory mechanism for reduced plasma adiponectin. In human subjects with T2D or impaired glucose tolerance, plasma adiponectin levels were decreased, while the expression of AdipoR1/R2 mRNAs were increased in skeletal muscle. In other studies, hyperinsulinemia and hyperglycemia were associated with reduced AdipoR1 mRNA levels in skeletal muscle and a decreased sensitivity to metabolic effects of GAdipoQ. On the other hand, hyperinsulinemia increased AdipoR2 mRNA levels and the sensitivity of muscle cells to FLAdipoQ. To conclude, the published literature seems to agree that plasma levels of adiponectin are decreased, while AdipoR1/R2 expression is generally increased in human skeletal muscle and liver of T2D and obese subjects, although the results are quite contradictory mainly when human and mouse subjects are compared.

In the case of plasma adiponectin, our own results (Figure 14B) failed to confirm the reduced secretion in db/db mice, while P17 administration had the tendency to increase the plasma adiponectin concentration when compared to healthy NMRI mice, but also in comparison with control db/db mice. These contradictory results could be explained by the measurement, by different authors, of adiponectin concentration in blood plasma with different dosage kits that could be more sensitive against various forms of adiponectin, i.e., FLAdipoQ, GAdipoQ, high molecular weight (HMW) adiponectin. Each one of them could present a different behavior in various pathological conditions and subject types, which could explain on the one hand the variable concentrations found in blood plasma and on the other hand the tissue reactivity to adiponectin signaling pathways. For instance, FLAdipoQ is known to activate AMPK in skeletal muscle, followed by FFA oxidation and glucose uptake. HMW oligomers of adiponectin are involved in insulin sensitivity, while their decline in blood plasma represents a risk factor in metabolic pathologies including the obesity. GAdipoQ on the other hand is involved in the improvement of adipocyte metabolism and function.

In our experimental conditions, we have observed that db/db mice present a significantly high expression ($p < 0.01$) of AdipoR1 in muscle (Figures 15A and 15A) and of AdipoR2 in liver (Figures 15B and 15B) when compared to healthy NMRI mice (Figures 15F and 16A/B). This high AdipoR1/R2 expression was independent of the diet composition (i.e., 60 kcal % or 10 kcal %), but it was relatively weaker in 10

kcal % fed mice and more variable in the liver of the same mice. The elevated AdipoR1/R2 expression in the skeletal muscle and liver of db/db mice could be related to their known insulin resistance and non-alcoholic steatohepatitis (NASH), the same as in T2D and obese patients. In these subjects, AdipoR1 but not AdipoR2 promoter activity seems to be enhanced by insulin via the PI3K/Foxo1 pathway. At the same time, our *in vivo* study confirms the *in vitro* results, which have shown that AdipoR1 and AdipoR2 expression is stimulated in HepaRG and C2C12 cells, when challenged with glucose and FFAC (Figure 7C).

In the db/db mice of our study, P17 administration induced a significant decrease of AdipoR1/R2 expression in muscle and liver, which presented the tendency to restore the level observed in healthy NMRI mice. A more variable effect was observed in the liver of db/db mice fed on a 10 kcal % diet, whereas the 60 kcal % diet seemed to potentiate the P17 effect. These results could corroborate our previous data obtained on HepaRG cells, where P17 induced an amplifier activation of AMPK when cells were incubated with a supplement of FFAC and glucose. This effect could be explained by the permissive activity of FFA on AMPK phosphorylation and on glucose uptake through the membrane translocation of GLUT4.

Regarding the cellular localization, AdipoR1 was homogenously distributed in the skeletal muscle fibers independently of the experimental group, including the healthy NMRI mice. AdipoR2 was homogenously distributed in the hepatocytes' cytoplasm and membrane of the db/db mice fed on a 60 kcal % diet, while AdipoR2 in mice fed on a 10 kcal % diet presented a predominant plasma membrane localization. P17 administration induced a more restrictive localization of AdipoR2 at the level of plasma membrane, which was similar to that of healthy NMRI mice mainly in the case of db/db mice fed on a 60 kcal % diet. The cytoplasmic distribution of AdipoR1/R2 could be related to their recycling and/or degradation in lysosomes after endocytosis, which is one of the mechanisms involved in the regulation of adiponectin signaling. It is thus possible that the high AdipoR1/R2 expression in db/db mice could be associated with an enhanced lysosome degradation and adiponectin signaling downregulation. As observed above on C2C12 cells, the glucose and FFAC supplement induced a striking increase of AdipoR1 expression, but also of the lysosome content, which could confirm the *in vivo* studies. It is also known that AdipoR1 and caveolin-1 associate at the plasma membrane, phenomenon that promotes AdipoR1 endocytosis within caveolae and adiponectin transmembrane signaling. This interaction is downregulated by high glucose and lipids concentrations, mainly because of the reduced caveolin-1 expression. In the case of AdipoR2, the sequence analysis revealed a potential

caveolin-binding motif in its transmembrane domain, which may be responsible of adiponectin signalosome formation. The membrane localization of AdipoR2 in the liver of db/db mice treated with P17 could be associated with caveolin binding and signalosome formation, phenomenon that could sustain the significant decrease of plasma triglycerides (Figure 14B) probably as a consequence of FFA oxidation in liver.

The cellular localization of AMPK-pT172 in skeletal muscle and liver presented a similar distribution with that of AdipoR1/R2, meaning that it was predominantly cytoplasmic in skeletal muscle and concentrated to the cell membrane in the case of liver, including in healthy NRM1 mice. The predominant cytoplasmic localization of AMPK-pT172 in the liver of db/db mice fed on a 60 kcal % diet was similar to that of AdipoR2 in the same tissue. The catalytic α subunit of AMPK comprises an activating phosphorylation site (T172) and occurs in two isoforms, $\alpha 1$ and $\alpha 2$. In mammalian cells, the AMPK $\alpha 2$ presents a nuclear and non-nuclear localization, while AMPK $\alpha 1$ is only found in non-nuclear fractions. Our results confirm thus the non-nuclear localization of AMPK $\alpha 1$, which is detected by the antibody employed in our study. In fact, the kinase LKB1, responsible of AMPK phosphorylation, is translocated from the nucleus to the cytosol when adiponectin binds to its receptors. This binding stimulates the interaction of the intracellular region of AdipoR1/R2 with the adaptor protein APPL1, which in turn immobilizes LKB1 that becomes able in this way to phosphorylate AMPK. It is therefore conceivable that AMPK is recruited in the proximity of cell membrane, where the complex APPL1/LKB1 is located. The second kinase responsible of AMPK phosphorylation, the CaMKK2, is indirectly activated by APPL1, which activates phospholipase C (PLC) that induces the release of Ca^{2+} from the endoplasmic reticulum subsequent to the production of inositol 1,4,5-trisphosphate (IP3). Thus, a cytoplasmic location of AMPK would be advantageous for an activation via the CaMKK2 pathway. After activation, AMPK phosphorylates several downstream targets, the global effect being to inhibit the ATP consuming pathways (i.e., protein and fatty acid synthesis), whereas the ATP producing pathways (i.e., glycolysis and FFA oxidation) are upregulated.

In our study, AMPK was significantly activated in the skeletal muscle and liver of db/db mice independently of the diet composition, although it was higher in mice fed on a 10 kcal % diet (Figures 16C and 16D). Concomitantly, this activation was more variable in the liver of the same mice. The lower level of AMPK activation in mice fed on a 60 kcal % diet could be associated to insulin resistance, as explained above for AdipoR1/R2. Moreover, the adiponectin resistance could also contribute to lower AMPK activation. It is known that obese subjects present a reduced AMPK activation that is not related to a reduced expression of AdipoR, which tends to be

higher. In fact, a 60% saturated fat diet can induce adiponectin resistance, as demonstrated by the failure of GAdipoQ to inactivate acetyl coenzyme A carboxylase (ACC) and stimulate FFA oxidation. Adiponectin resistance could thus explain the high plasma triglyceride concentration observed in db/db mice (Figure 14B), which is certainly the consequence of a reduced FFA oxidation.

The same as in the case of AdipoR1/R2, peptide P17 induced a significant decrease of AMPK activation ($p < 0.05$) in skeletal muscle and liver of db/db mice fed on a 60 kcal % diet as compared to the control group. Although the effect on AMPK activation was not significantly different in db/db mice fed on a 10 kcal % diet when compared to the control group, the general tendency was to reduce AMPK phosphorylation at a level closer to the healthy NMRI mice, which could be a sign of metabolic improvement. Furthermore, AMPK-pT172 in liver was restricted to the cell membrane of hepatocytes in db/db mice treated with P17, which could be related to its activation by LKB1. The significant decrease of plasma triglyceride concentration in both groups of mice treated with P17 (Figure 14B) could confirm the AMPK activation and FFA oxidation. As explained above, these results seem to confirm the striking AMPK activation produced by P17 in HepaRG cells incubated with a supplement of FFAC and glucose, effect that could be explained by the allosteric modulation of AMPK in the presence of FFA, which potentiates its phosphorylation by LKB1.

PPAR α activation (PPAR α -pS12) in liver followed the same evolution as that of AMPK phosphorylation among the experimental groups, meaning that db/db mice presented a higher level of PPAR α phosphorylation independently of the diet composition (Figures 15E, 15F and 16E), although those fed on a 10 kcal % diet were characterized by the highest level of PPAR α activation, at least from the viewpoint of its phosphorylation. Moreover, PPAR α -pS12 was distributed both in the nuclei and cytoplasm of hepatocytes. This distribution was mostly observed in the case of db/db mice fed on a 10 kcal % diet, but also in those fed on a 60 kcal % diet.

PPAR α is a transcription factor that regulates the expression of genes involved in FFA oxidation, ketogenesis, lipid transport and gluconeogenesis. Its dysregulation has been involved in the etiology and pathogenesis of diabetes, obesity, hyperlipidemia, atherosclerosis, cancer, inflammation etc. In three obese mouse models (i.e., ob/ob, db/db, 5-HT2cR), the PPAR α mRNA expression was increased by 2- to 3-fold as compared to healthy lean mice. It has also been demonstrated that PPAR α and PPAR γ are dynamically shuttled between nucleus and cytoplasm, although they are present constitutively and predominantly in the nucleus. Their shuttling is possible due to the harboring of at least two nuclear

localization signals (NLS) and of two nuclear export signals (NES) that are recognized by distinct importins and exportins. Their subcellular localization seems to be regulated by various external and internal signals, such as their own ligands (i.e., fatty acids derived from diet or intracellular signaling pathways) and the intracellular Ca^{2+} concentration. In the cytoplasm, PPAR α has been found to interact with centrosome-associated protein CAP350, and together they were localized at the level of centrosome and in a branched filamentous network of intermediary filaments distributed throughout the cytoplasm. It has been suggested that PPAR α recruitment to the centrosome could play a role in its turnover via the ubiquitination and proteolysis at the level of a 26S proteasome located in the centrosome.

Consequently, our results seem to corroborate the published data by showing a high level of PPAR α activation and probably expression in the liver of our db/db mice. Despite of this high level of PPAR α activation, its subcellular localization was both nuclear and cytoplasmic, which could correspond to a mechanism of PPAR α downregulation, eventually by proteolysis. This downregulation could explain the high level of plasma triglyceride concentration in both groups of control db/db mice, independently of the diet composition (Figure 14B).

The treatment of mice with peptide P17 induced a significant decrease of PPAR α activation (and probably expression) in db/db mice fed on a 60 kcal % diet ($p < 0.01$ vs. control db/db mice), its level being identical to that in healthy NMRI mice (Figure 16E). In db/db mice fed on a 10 kcal % diet, the level of PPAR α -pS12 was also decreased by the treatment with P17, although the results were more variable and did not attain significance. At the same time, PPAR α -pS12 was predominantly located in the hepatocytes' nuclei in the liver of mice treated with P17, which was comparable to its subcellular localization in the liver of healthy NMRI mice. This nuclear localization of PPAR α -pS12 could signify that its transcriptional activity involved in lipid metabolism could have been restored in the range of controls, fact that is corroborated by the plasma concentration of triglycerides in db/db mice treated with P17, which was identical to that in healthy NMRI mice (Figure 14B).

In summary, our results revealed that db/db mice present high levels of AdipoR1/R2 expression in skeletal muscle and liver, which might be related to their known insulin resistance and NASH, phenomena that are characteristic to T2D and obese subjects. The subcellular localization of AdipoR1/R2 was homogenous in the cytoplasm and membrane of skeletal muscle fibers and liver, the cytoplasmic distribution being probably related to their lysosome degradation. The treatment of db/db mice with P17 presented the tendency to restore the level of AdipoR1/R2 expression in the range of healthy NMRI mice. Moreover, AdipoR2 was more

restricted at the level of plasma membrane of hepatocytes, where it could have been associated to caveolin and contribute to signalosome formation. The AMPK-pT172 was also present in higher quantities in the skeletal muscle and liver of db/db mice as compared to healthy NMRI mice. However, AMPK activation was lower in mice
5 fed on a 60 kcal % diet, possibly in conjunction with adiponectin resistance, characteristic to obese subjects. The administration of P17 induced the return of AMPK-pT172 level in the range of healthy NMRI mice, mainly in the case of db/db mice fed on a 60 kcal % diet, probably in relationship with allosteric modulation of AMPK by FFA. Moreover, P17 induced the restriction of AMPK at the level of
10 hepatocyte membrane, where it could be phosphorylated by LKB1. PPAR α in liver was also highly activated by phosphorylation (PPAR α -pS12) independently of the diet composition, but it was located both in the nuclei and cytoplasm of db/db mice. The cytoplasmic distribution could be related to its downregulation by proteolysis at the level of 26S proteasome and explain the low level of lipid oxidation as shown by
15 the high plasma triglyceride concentration in these mice. The P17 administration presented the tendency to return the level of PPAR α activation in the range of healthy NMRI mice and restricted its subcellular distribution to the nucleus, where it could regulate the expression of genes involved in lipid metabolism. This regulation is furthermore confirmed by the diminution of plasma triglyceride
20 concentration at the level of healthy NMRI mice.

4.3. The effects on cell survival and fatty liver disease

Apoptosis is an important feature of T2D and metabolic syndrome, dealing with various cell types such as pancreatic beta and alpha cells, but also the hepatocytes. In fact, the death of beta cells by apoptosis is characteristic to both
25 T1D and T2D, where this is triggered by the signaling pathways of interleukin (IL)-1 β , nuclear factor (NF)- κ B and Fas. However, if the beta cell failure is induced in T1D by an autoimmune attack produced by macrophages and cytotoxic T-cells, their death in T2D is caused by the chronic exposure to high glucose and FFA concentrations. It is also known that the response of alpha cells to the variable levels
30 of glycaemia is compromised in both T1D and T2D. Alpha cell area is moreover reduced in obese mice fed a high fat diet, in conjunction with alpha cell hypotrophy, increased apoptosis and decreased proliferation. In addition to pancreatic beta and alpha cell dysfunction, the non-alcoholic fatty liver disease (NAFLD) is a common complication of obesity and T2D. The NAFLD can occur in two clinical presentations,
35 the non-alcoholic fatty liver (NAFL), characterized by hepatic inflammation, and NASH, which is characterized by steatosis and hepatocyte apoptosis, and is related to insulin resistance.

The proliferative and anti-apoptotic activities of adiponectin were already demonstrated in various tissues, such as the heart, liver and pancreas. Although the mechanisms involved in cell survival and protection against apoptosis were not completely elucidated, the role of PI3K/Akt and ERK pathways as well as of the sphingosine-1-phosphate (S1P) receptor was reported in the specialized literature. The activation of Akt and ERK pathways is furthermore involved in the stimulation of insulin gene expression and secretion by pancreatic beta cells. Adiponectin also exerts hepato-protective actions by inactivating ACC, which reduces lipid synthesis and enhances the FFA oxidation, and downregulates the expression of sterol regulatory element-binding protein 1c (SREB-P1c), a transcription factor involved in lipid synthesis. The activation of PPAR α is moreover responsible for the FFA oxidation. All these pathways contribute to an enhanced fat oxidation, reduced lipogenesis and prevention of hepatic steatosis.

In the light of these pathological aspects of T2D and the protective effects exerted by adiponectin, we have hypothesized that peptide P17 could prevent cell apoptosis in pancreatic islets and liver of db/db mice if it binds correctly to AdipoR1/R2 and triggers the adiponectin pathway. Apoptotic cells were thus detected in these tissues by the immunofluorescent staining of activated caspase-3, while NASH was observed in liver by IHC after its Masson's Trichrome staining.

In the case of liver, control db/db mice presented a significantly increased ($p < 0.05$, $p < 0.01$ vs. healthy NMRI mice) staining of activated caspase-3 (Figure 17), independently of the diet composition. The staining level was in the range of positive control represented by apoptotic livers from Balb/c mice injected with anti-Fas antibody and confirmed that T2D developed by db/db mice is indeed characterized by liver apoptosis probably induced by NASH as described by literature.

The two groups of db/db mice treated with P17 have shown a significantly decreased staining of activated caspase-3 (Figure 17) when compared to control db/db groups ($p < 0.01$) or to positive control represented by apoptotic liver ($p < 0.05$, $p < 0.01$), although the level remained significantly superior ($p < 0.01$) to healthy NMRI mice. The inhibition of liver apoptosis induced by P17 corroborates with its ability to restore AMPK and PPAR α activation and cellular localization in the range of healthy controls (Figures 15 and 16). The regulation of these two signaling proteins by P17 was associated with a significant decrease of plasma triglyceride concentration (Figure 14B), which was identical to healthy NMRI mice, and confirmed the liver lipid oxidation that is a condition for NASH treatment. The reduced steatohepatitis in db/db mice treated with P17 was furthermore confirmed by the Masson's Trichrome staining (Figure 18), which revealed the tendency of liver morphology to recover an aspect comparable to that of healthy tissue, effect that

was more evident for db/db mice fed on a 60 kcal % diet. The liver of control db/db mice treated with PBS shows clear steatosis with hepatocellular ballooning. However, no inflammatory infiltration or liver fibrosis could be observed in this animal model.

5 Pancreatic islets did not show any apoptotic events associated with beta cells in either of the experimental groups, as confirmed by the absence of colocalization between activated caspase-3 immunoreactive cells and cells stained for insulin (Figure 19). Conversely, alpha cells were all immunoreactive for activated caspase-3 (Figure 20), which perfectly co-localized with glucagon in all experimental groups
10 including healthy NMRI mice. We have also identified a perfect colocalization between activated caspase-3 and alpha cells in the pancreas of healthy C57BL6/J mice (data not shown), which confirms that alpha cell apoptosis is not a feature characteristic to healthy NMRI mice. The literature published so far is mainly focused on beta cell apoptosis in diabetes subjects, the studies being largely performed *in*
15 *vitro* on isolated pancreatic islets or beta cells. Consequently, the reported data cannot be used to compare or interpret our results.

 We have therefore calculated the percentage of beta cells over alpha cells per pancreatic islet based on their immunoreactivity for insulin and glucagon (Figure 20B). These results revealed that control db/db mice treated with PBS presented a
20 decreased percentage of beta cells in conjunction with an increased percentage of alpha cells as compared to healthy NMRI mice. At the same time, the total number of beta cells per islet have shown the tendency to be larger than in healthy NMRI mice, confirming their capacity to compensate for an increased metabolic load and insulin demand as demonstrated in rodents. Moreover, the increased percentage of
25 alpha cells may belong to a compensatory mechanism of adaptation, which could originate from the necessity to equilibrate glycemia under a high insulin secretion by the enlarged beta cell mass, at least at the beginning of pathological process. The death of alpha cells by apoptosis may contribute to a recycling mechanism, meant to renew these cells under the challenge of metabolic stress.

30 The treatment of db/db mice with peptide P17 restored the ratio of beta cells over alpha cells in the range of healthy NMRI mice, being significantly different ($p < 0.05$) from PBS treated db/db mice. The beta cell number per pancreatic islet has also significantly increased ($p < 0.01$, $p < 0.05$) in comparison with healthy NMRI mice, probably as a mechanism intended to compensate for the increased metabolic
35 demand, triggered by the activation of adiponectin pathway. Moreover, adiponectin triggers PI3K/Akt and ERK pathways, which are responsible of cell survival and proliferation.

To conclude, these findings have shown that liver steatosis is associated with apoptotic hepatocyte death in db/db mice, characteristic to NASH as described by literature, although leucocyte infiltration and liver fibrosis could not be observed in our animal model. Treatment with P17 has significantly reduced both steatohepatitis and apoptosis, possibly in relationship with its ability to restore the physiological activity and cellular localization of AMPK and PPAR α , and to enhance lipid oxidation as demonstrated by the reduced plasma triglyceride concentration.

In the case of pancreatic islets, no apoptotic beta cells could be observed in db/db mice, confirming the published literature, which failed to evidence a significant increase of apoptotic cells in pancreatic islets of 5- to 24-week old db/db mice. However, the apoptotic death of alpha cells was identified in the present study both in db/db and healthy mice, phenomenon that deserves to be considered and investigated in future studies of diabetes research. On the other hand, the total number of beta cells per islet was larger in control db/db mice as compared to healthy NMRI mice, the same as the percentage of alpha cells per islet. Therefore, the beta cell hyperplasia, characteristic to at least early stages of T2D and obese subjects, is accompanied by a proportional increment of alpha cell number, which could represent a compensatory mechanism contributing to glycemia homeostasis upon insulin hypersecretion. Alpha cells furthermore present the highest replication rate under basal conditions or mitogen stimulation in comparison to beta, delta and PP cells, which could be associated with a shorter life-span and contribute to the islet cell plasticity. The ratio of beta cells over alpha cells was restored by peptide P17 to the level characteristic to healthy mice, while beta cell mass was augmented, phenomenon that could be explained by the activation of adiponectin pathways involved in metabolism, but also in cell proliferation and survival.

Conclusions

It is now generally accepted that obesity is a chronic disease that can lead to serious complications (i.e., diabetes, cardiovascular disease and cancer) in the absence of an adequate treatment, which remains to be developed. The World Health Organisation (WHO) has shown that worldwide obesity has more than doubled since 1980, which contributes to a substantial increased morbidity and mortality, as well as to an increased health care costs.

Adipokines are among the most important molecular actors in the pathophysiology of obesity-linked disorders by their ability to regulate inflammatory and metabolic processes. Adiponectin is an adipokine secreted by the adipose tissue, which decreases in diabetic and obese patients. Its replenishment has an anti-

diabetic effect by improving insulin sensitivity and cell survival, including that of beta cells. Adiponectin has been furthermore reported to produce pleiotropic beneficial effects in various pathologies, such as obesity, MS, CVD, fatty liver disease and liver fibrosis.

5 Adiponectin has two main receptors, AdipoR1 and AdipoR2, which have seven transmembrane domains that are structurally and functionally distinct from G protein-coupled receptors. AdipoR1 activates AMPK pathways that regulate the inhibition of gluconeogenesis, increase fatty acid oxidation and glucose uptake. AdipoR2 activates PPAR α pathways, which stimulate energy dissipation by
10 increasing fatty acid oxidation and inhibit oxidative stress and inflammation. These molecular pathways contribute to an increased insulin sensitivity and to a reduced risk of incident T2D in apparently healthy individuals.

 In the present work, a peptide agonist of AdipoR1/AdipoR2 was developed that modulates adiponectin signaling pathways and regulate glucose and lipid
15 metabolism, showing a relevance for T2D and obesity associated pathologies. The selected peptides bind to a 12-mer sequence comprised in the C-terminal domain of AdipoR1/AdipoR2, which is homologous for both receptors, in humans and mice. The biochemical properties of amino acid residues are identical to AdipoR1-12C and similar to AdipoR2-12C and seem to reveal an ability to bind AdipoR in the close
20 proximity of cell membrane due to the equilibrated proportion of hydrophilic (58.4%) and hydrophobic (41.7%) residues. The 20 hours theoretical half-life and its homology with proteins involved in insulin sensitivity, cell proliferation, the defense against cell stress and control of protein folding suggest that these peptides could be a putative pharmacological candidate for *in vivo* modulation of AdipoR
25 signaling.

 The binding of the peptides to AdipoR1/R2 was confirmed by immunofluorescent colocalization on tissue sections of mouse skeletal muscle and liver and on human pancreas, which suggest that the latter could recognize both receptors. In skeletal muscle, AdipoR1 is principally expressed by fast-twitch type
30 II fibers, while AdipoR2 is expressed by both fast and slow fibers although its distribution in slow-twitch type I fibers is more heterogenous. These results seem to corroborate the pathways regulated by AdipoR1/R2, considering that AdipoR1 is mostly involved in the regulation of glucose metabolism (characteristic to type II fibers), whereas AdipoR2 regulates the lipid metabolism (characteristic to type I
35 fibers). The peptides were identified in both fiber types, while their binding in pancreas was restricted to pancreatic islets, with no interaction with exocrine pancreas. In the liver, the peptides have been noticed at the level of cell membranes.

Regarding the AdipoR1/R2 expression and signaling, we have observed that high glucose concentration stimulates AdipoR1 expression in C2C12 cells, which is associated with AMPK activation by phosphorylation. In HepaRG cells, FFAC seem to represent an inducer of AdipoR2 expression and of AMPK activation. Although these results need to be confirmed by future studies of RT-qPCR and Western blot, they corroborate the reported allosteric modulation of AMPK by FFA, which positively regulates AMPK phosphorylation by LKB1. Moreover, AMPK activation by high glucose concentration in C2C12 cells could be associated with membrane translocation of GLUT4 to assist glucose uptake by muscle cells.

On the other hand, the co-incubation of both cell types with high glucose concentration and FFAC seems to stimulate AdipoR1/R2 expression, but AMPK phosphorylation is decreased. Future studies should evaluate the PPAR α activation in muscle cells and hepatocytes, in culture conditions comprising both high glucose concentration and FFAC. PPAR α is a transcription factor that regulates lipid metabolism and glucose uptake and is activated by AdipoR2. The enhanced SDHA expression observed in these culture conditions could be related to PPAR α activation.

Among the candidate AdipoR1/R2 agonist peptides, P17 (seq ID n° 5) emerged as the most potent activator of AMPK phosphorylation in HepaRG and C2C12 cells. In agreement with AMPK phosphorylation, the cells induced with P17 presented a significant activation of GK and SDHA expression that could be related to its agonist AdipoR1/R2 activity. Activated AMPK increases FFA uptake and oxidation and stimulates GK expression and activity. Moreover, the activation of AMPK pathway by P17 seems to be sustained by a diminished lysosomal degradation of AdipoR1 combined with an improved colocalization with caveolin-1, known to be critical for signalosome formation and transmembrane signaling.

The AdipoR1/R2 agonist activity of P17 was subsequently studied *in vivo* on the db/db mouse model of T2D fed *ad libitum* on a Western high-fat diet (60 kcal; mice aged of 6 weeks) or on a standard chow diet (10 kcal; mice aged of 7 weeks). Considering that *Mus musculus* is a strongly nocturnal species [69], we have decided to treat them with P17 during the evening time, which should correspond to the fasting period, just before the beginning of the feeding and locomotion time. Our results have shown that P17 administered for four consecutive days promoted the body weight stabilization, the tendency of glycemia to be decreased, the drop of plasma triglyceride concentration in the range of healthy NMRI mice and the slight increase of adiponectin secretion. The effect on glycemia and plasma adiponectin was unexpectedly more evident in the case of db/db mice fed on a 60 kcal diet, and may be explained either by the one-week difference of age between the two groups

of mice (i.e., 6 weeks for 60 kcal diet vs. 7 weeks for 10 kcal diet) or by the effect of FFA on AMPK activation and glucose uptake.

The high AdipoR1/R2 expression found in skeletal muscle and liver of db/db mice confirmed the published literature in the case of human T2D and obese subjects. AdipoR1/R2 were generally homogenously distributed in the cytoplasm and plasma membrane of skeletal muscle fibers and hepatocytes, the cytoplasmic localization being probably the consequence of their downregulation via lysosome degradation. The high AdipoR1/R2 expression could thus represent a homeostatic mechanism meant to compensate for lysosome degradation. Although AMPK and PPAR α were significantly activated in skeletal muscle and liver of db/db mice, the high plasma triglyceride concentration suggests that AdipoR1/R2 intracellular signaling was inactivated by different molecular mechanisms, i.e. adiponectin resistance, AMPK and PPAR α turnover by ubiquitination and proteolysis. This hypothesis is furthermore sustained by the liver steatosis and apoptosis observed in these mice.

In mice treated with P17, AdipoR1/R2 expression presented the tendency to restore the level observed in healthy NMRI mice. Moreover, AdipoR2 in liver was more restricted to the plasma membrane in a similar manner as in healthy NMRI mice, which could be explained by caveolin binding and signalosome formation and contribute to the striking decrease of plasma triglycerides. Future studies should confirm this hypothesis by AdipoR2 colocalization with caveolin-1, which is known to play major roles in hepatic lipid and glucose metabolism [71]. The AMPK-pT172 and PPAR α -pS12 were also reduced by the P17 treatment, presenting the tendency to return to the level and cellular distribution characteristic to healthy NMRI mice. In liver, AMPK-pT172 was furthermore restricted to the cell membrane, while PPAR α -pS12 was concentrated in nuclei, restoring the subcellular distribution observed in healthy NMRI mice. The membrane location of AMPK-pT172 could be related to its activation by LKB1, whereas nuclear shuttling of PPAR α -pS12 is associated to the expression of genes involved in lipid metabolism. The regulation of adiponectin signaling pathway is moreover demonstrated by the significantly reduced steatohepatitis and liver apoptosis in db/db mice treated with P17. In the pancreas, P17 restored the ratio of beta cells over alpha cells in the range of healthy NMRI mice and increased the beta cell mass, probably via the activation of adiponectin pathways responsible of cell proliferation and survival.

Sequence ID's

SEQ ID n°1: Val His Trp Asp Phe Arg Gln Trp Trp Gln Pro Ser

SEQ ID n°2: Ala His Ala His Thr Asn Trp Thr Ser Trp Trp Glu

55

SEQ ID n°3: Asp Leu Val Ser Trp Ala Gly Ser Gly Lys Lys His

SEQ ID n°4: Ala Asp Trp Tyr His Trp Arg Ser His Ser Ser Ser

SEQ ID n°5: Ile Pro Asn Tyr Ser Met Gln Ser Arg Glu Tyr Arg

SEQ ID n°6: His Tyr Arg Pro Phe Thr Gln Glu His Arg Val Thr

5 SEQ ID n°7: His Ser Phe Lys Gly Trp Asp Trp Pro Arg Leu Arg

SEQ ID n°8: Gly Trp Lys Ser His Glu Pro Lys Gly His Gly Ser

SEQ ID n°9: His Ser Phe Lys Trp Leu Asp Ser Pro Arg Leu Arg

SEQ ID n°10: Gly Ala Tyr Thr Ser Trp Arg Thr Ser Thr Asn Ala

SEQ ID n°11: Glu His Leu His Ala Ser Trp Asn Phe Ser Ser Gly

10 SEQ ID n°12: Tyr Asp Val Pro Asn Lys Ser Trp Arg Thr Ser Trp

SEQ ID n°13: His Phe Tyr Gly Val Ser Asn Leu Gln Glu Phe Arg

CLAIMS

1. A peptide capable of binding to adiponectin receptors 1 or 2, characterized in that said peptide has at least 95% sequence identity to an amino acid sequence chosen from SEQ ID 2 to 3 or 5 to 12.
2. Peptide according to claim 1, characterized in that said peptide has at least 99% sequence identity to an amino acid sequence chosen from SEQ ID 2 to 3 or 5 to 12.
3. Peptide according to claim 1 or 2, wherein said peptide is a peptide has an amino sequence chosen from SEQ ID 2 to 3 or 5 to 12.
4. Peptide according to any of the claims 1 to 3, wherein said peptide is able to bind to a region of the adiponectin receptors 1 or 2, wherein said region comprises an amino acid sequence according to SEQ ID 13.
5. Peptide according to any of the previous claims, wherein said peptide is an agonist of adiponectin receptors 1 or 2.
6. A region within AdipoR1 and AdipoR2, wherein said region has an amino acid sequence according to SEQ ID n°13.
7. Molecule able to bind to the region of claim 6 and able to modulate receptor signaling.
8. Molecule according to claim 12, wherein said molecule is a protein, a peptide or a compound and wherein AdipoR1 and/or AdipoR2 signaling is activated upon binding of said protein, peptide or compound to said AdipoR1 and/or AdipoR2.
9. Composition comprising one or more peptides according to the previous claims 1 to 5 or one or more molecules according to claim 7 or 8.
10. Composition according to claim 9, wherein said composition is formulated to be administered intraperitoneally, orally, intramuscularly, subcutaneously or intravenously.
11. Composition according to claim 9 or 10, wherein said composition is a liquid or wherein said composition is formulated as a tablet, a powder or a capsule.
12. Composition according to any of the claims 9 to 11 wherein said composition is a food supplement, preferably a dietary food supplement, or a pharmaceutical composition.
13. Peptide according to any of the claims 1 to 5 or composition according to any of the claims 9 to 12 for therapeutic use.
14. Peptide according to any of the claims 1 to 5 or a composition according to any of the claims 7 to 13 for use in the treatment or control of Type 2

Diabetes, the treatment of systemic disease or for use in weight control in a subject.

15. Peptide or composition for use according to claim 13 or 14, wherein said peptide is administered to a subject at a dose of between 10 nmol and 100 μ mol/kg.
16. Peptide having at least 95% sequence identity to an amino acid sequence chosen from SEQ ID n°1 to 12 or a composition comprising said peptide for use in the treatment or control of Type 2 Diabetes, the treatment of systemic disease or for use in weight control in a subject.
17. Peptide or composition for use according to claim 16, wherein said peptide is administered to a subject at a dose of between 10 nmol and 100 μ mol/kg.

Figures

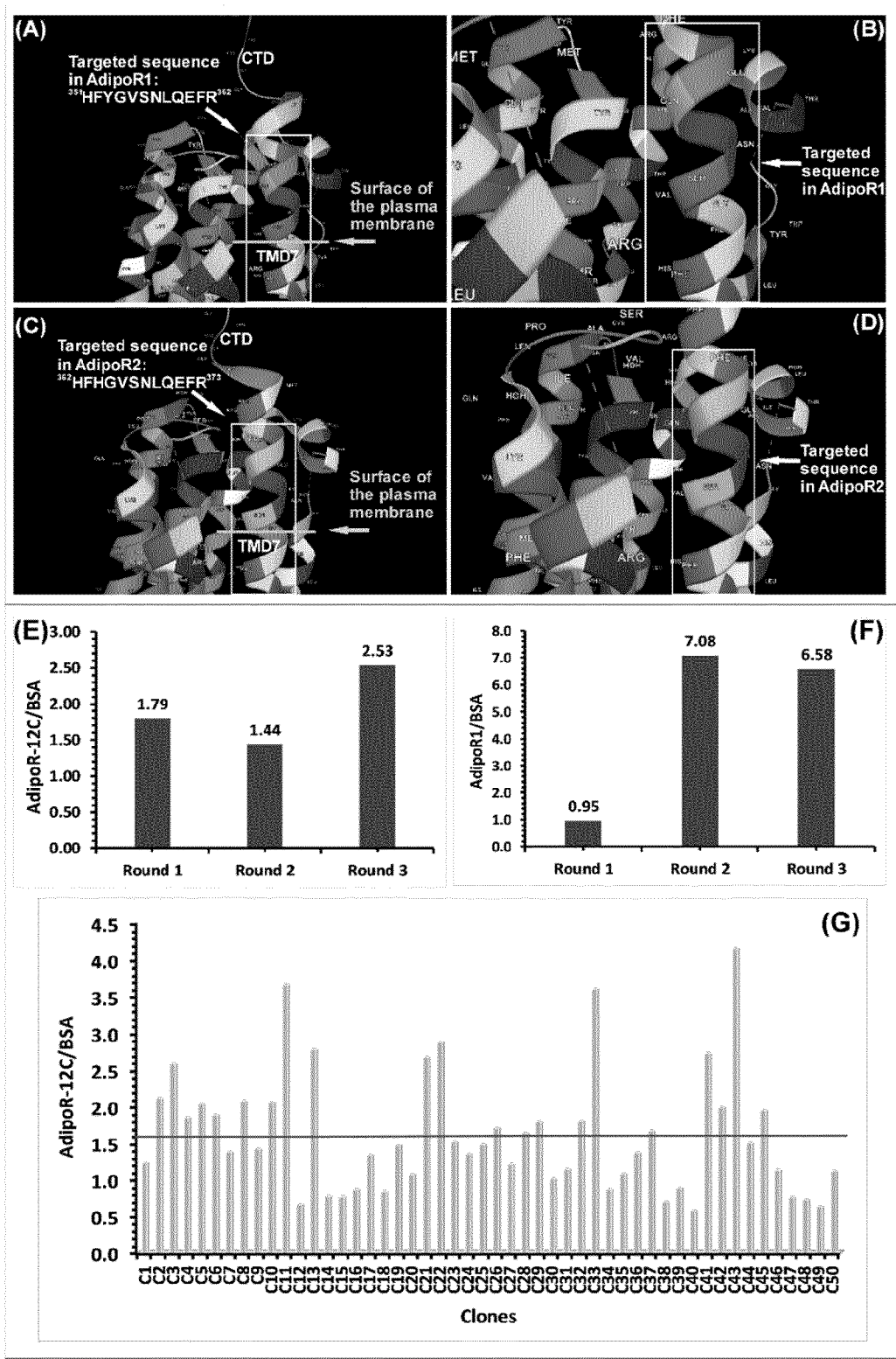


Figure 1

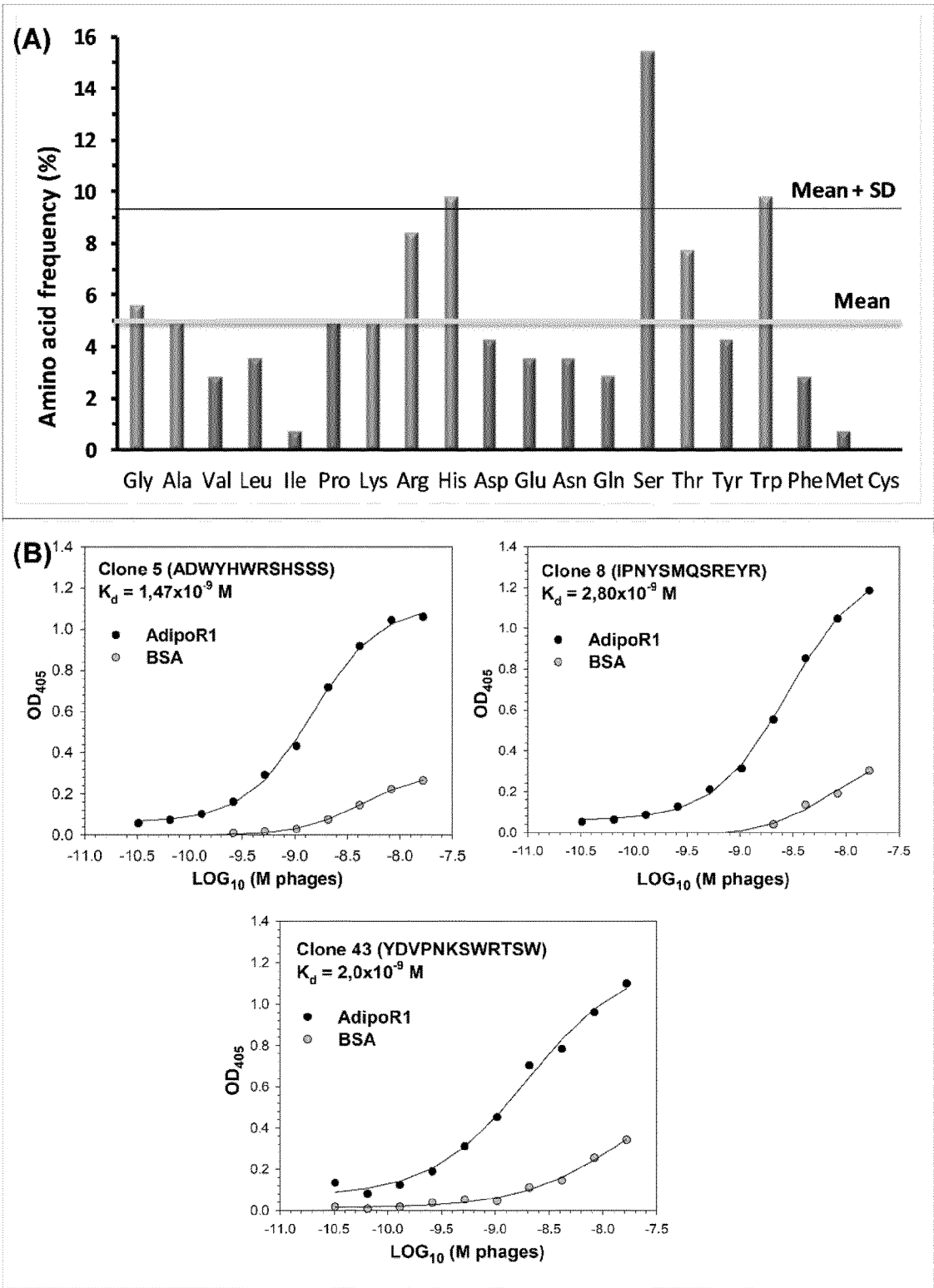
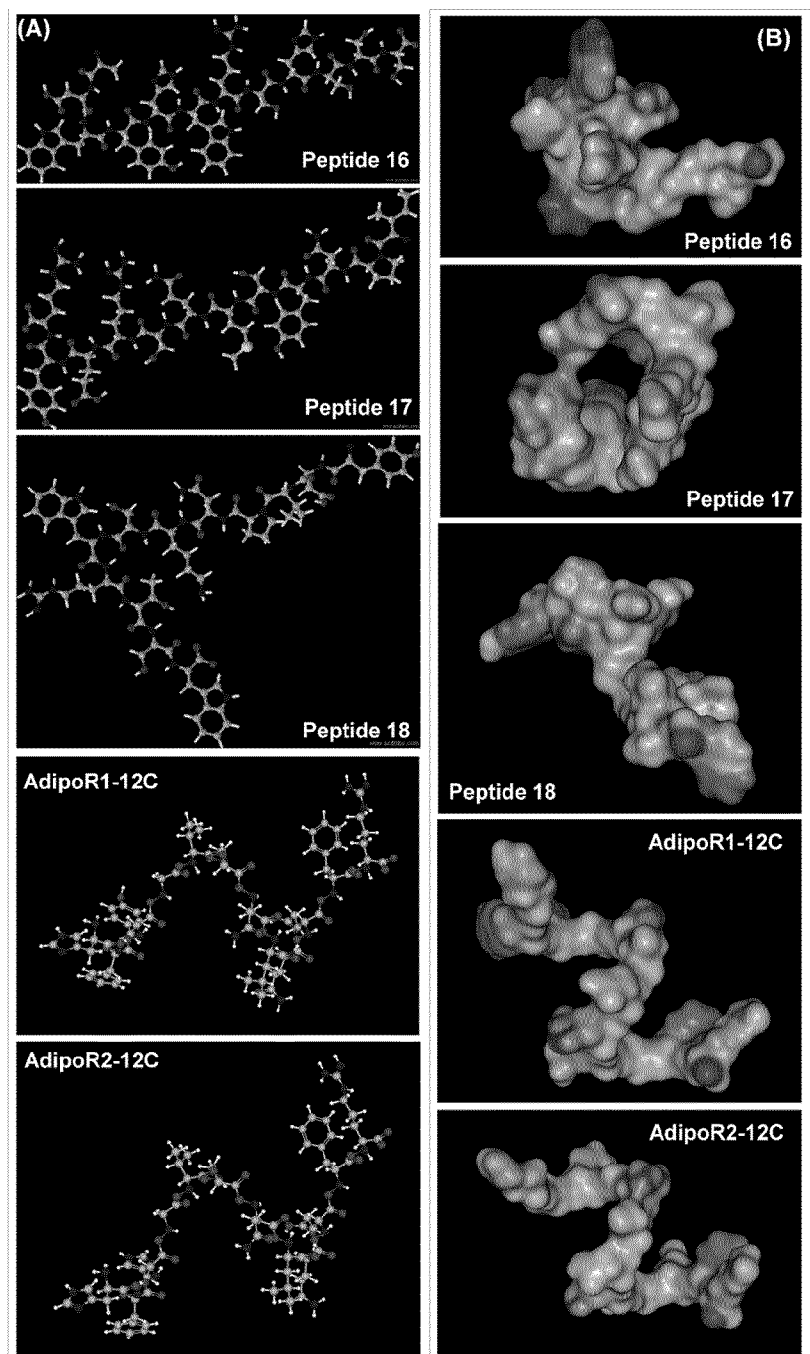


Figure 2

**Figure 3**

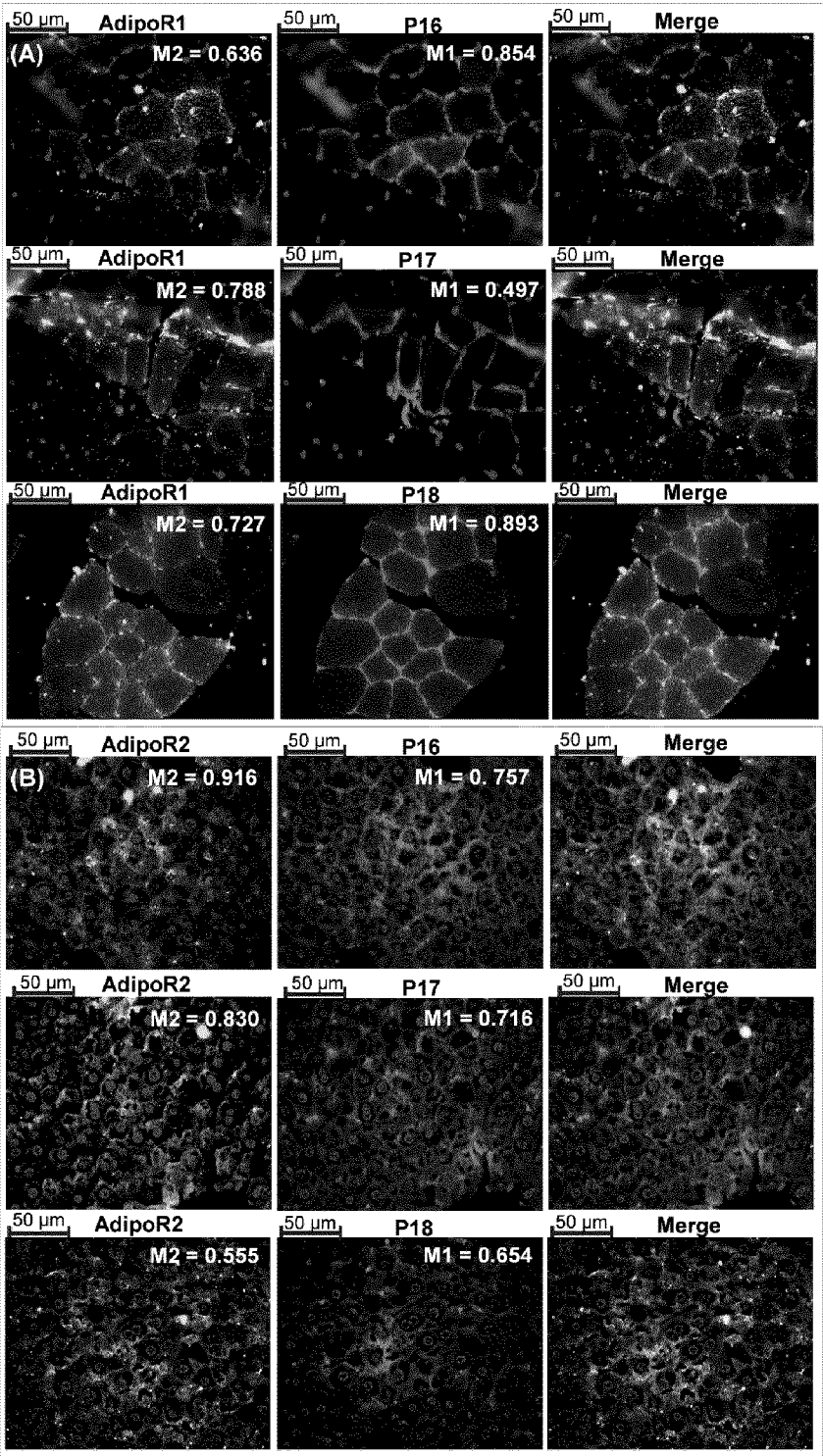


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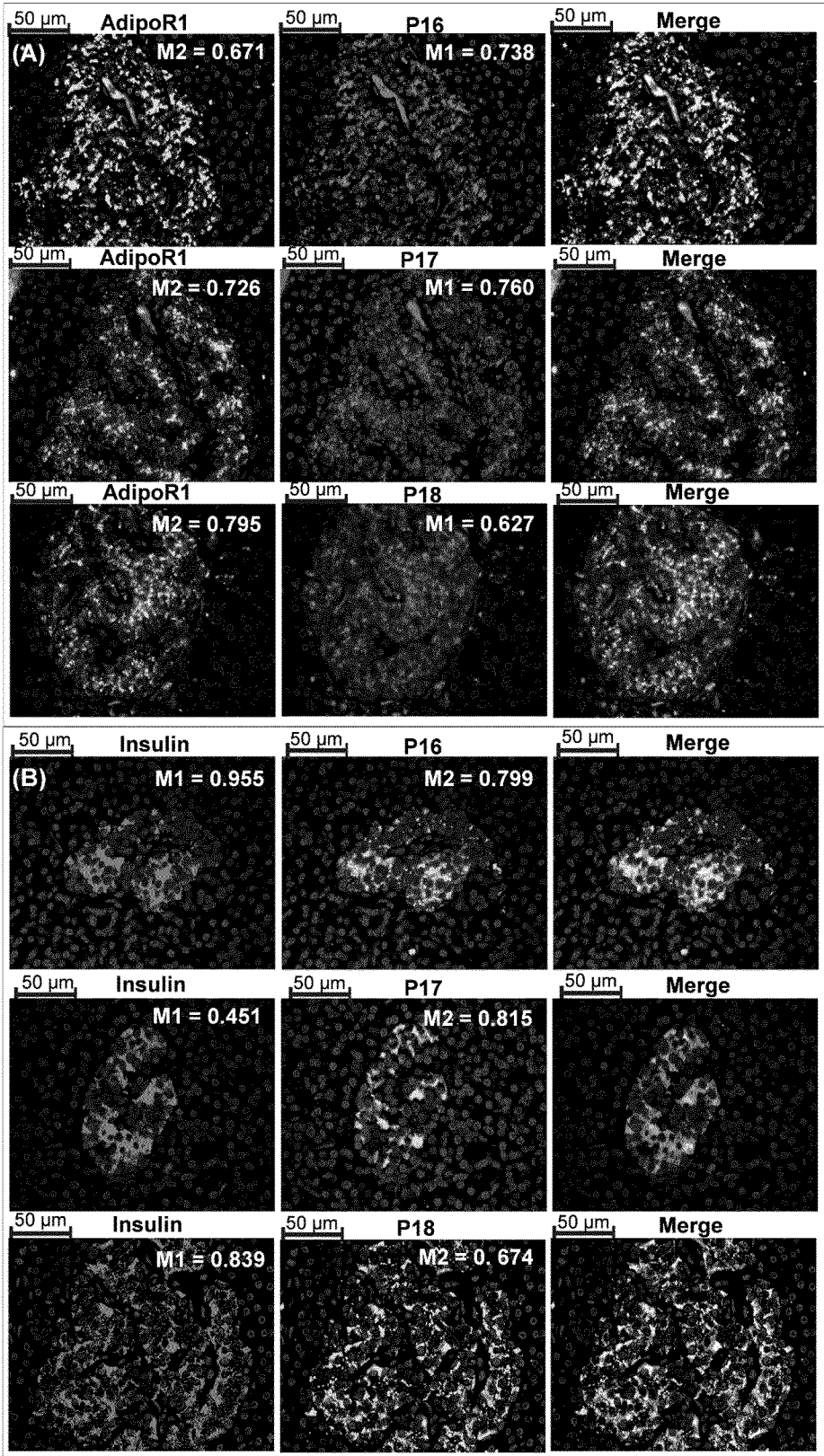


Figure 5

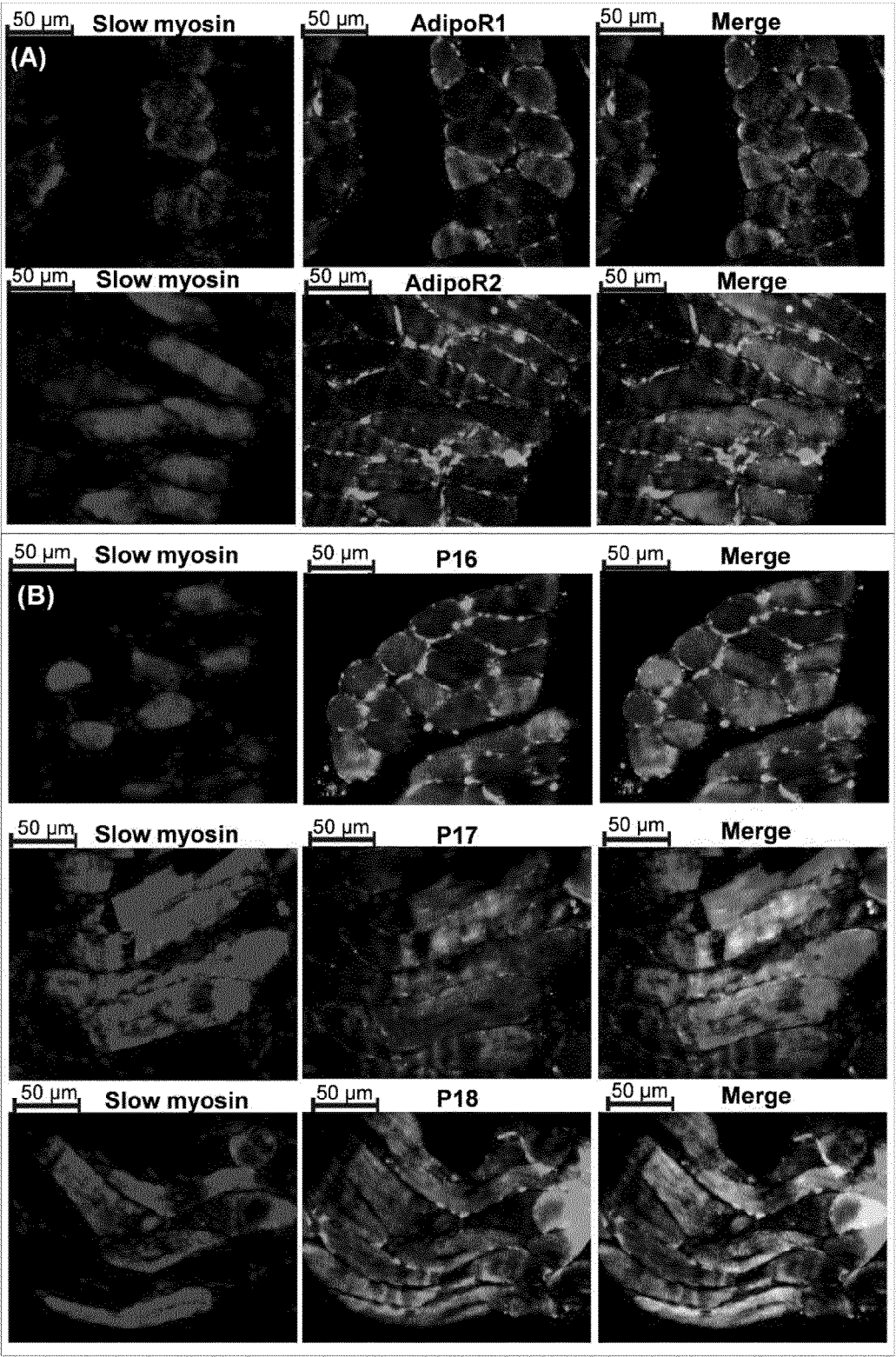


Figure 6

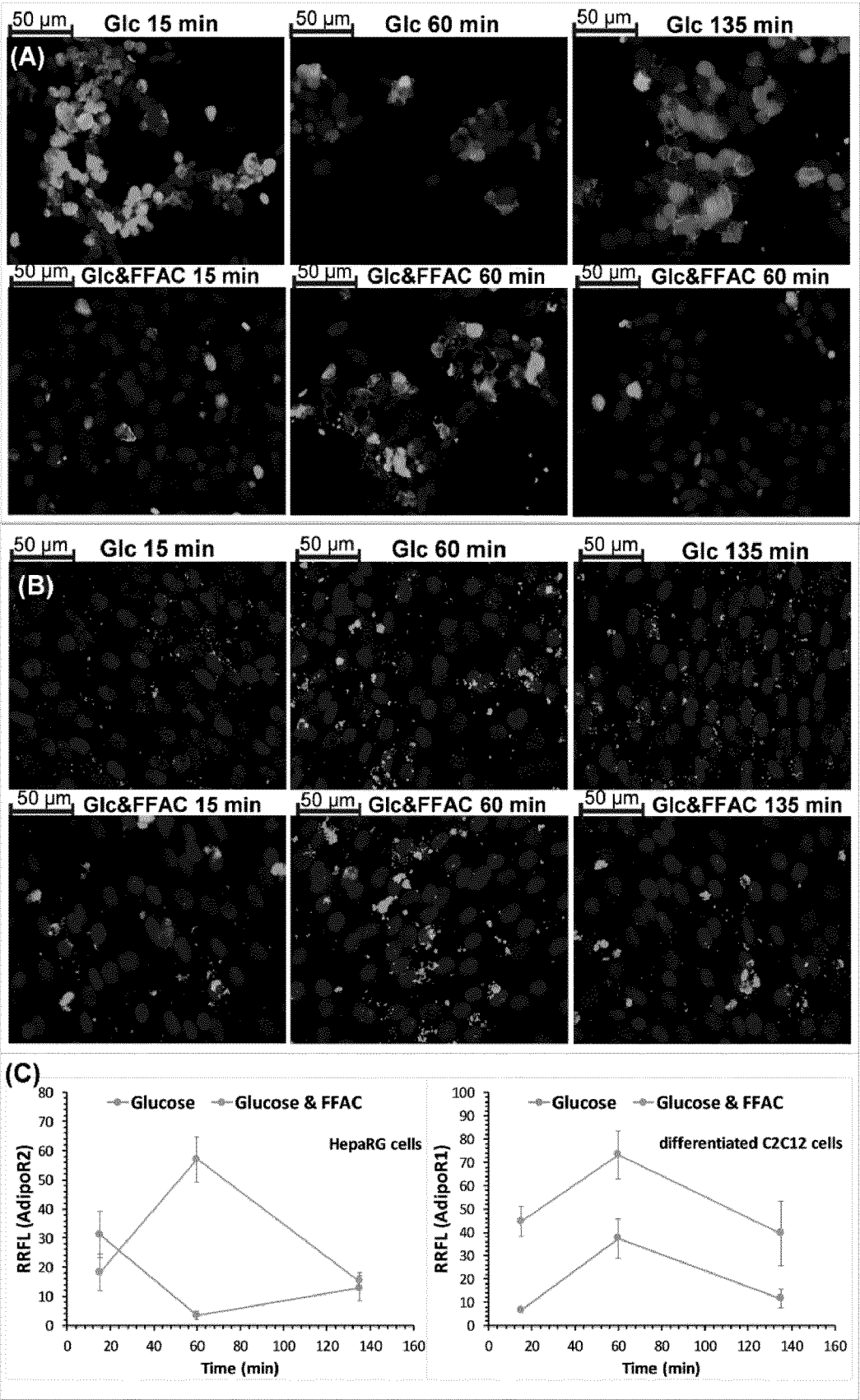


Figure 7

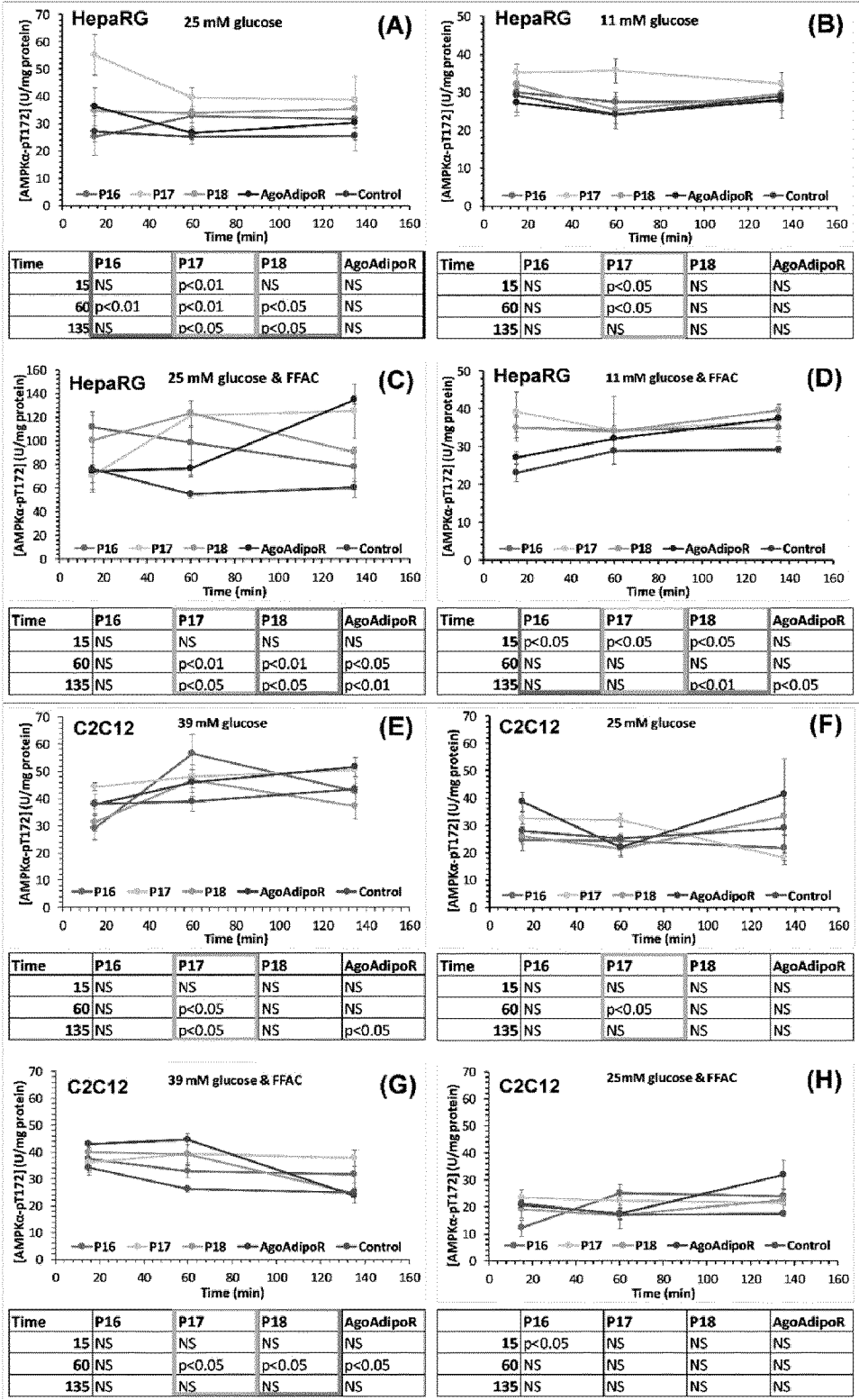


Figure 8

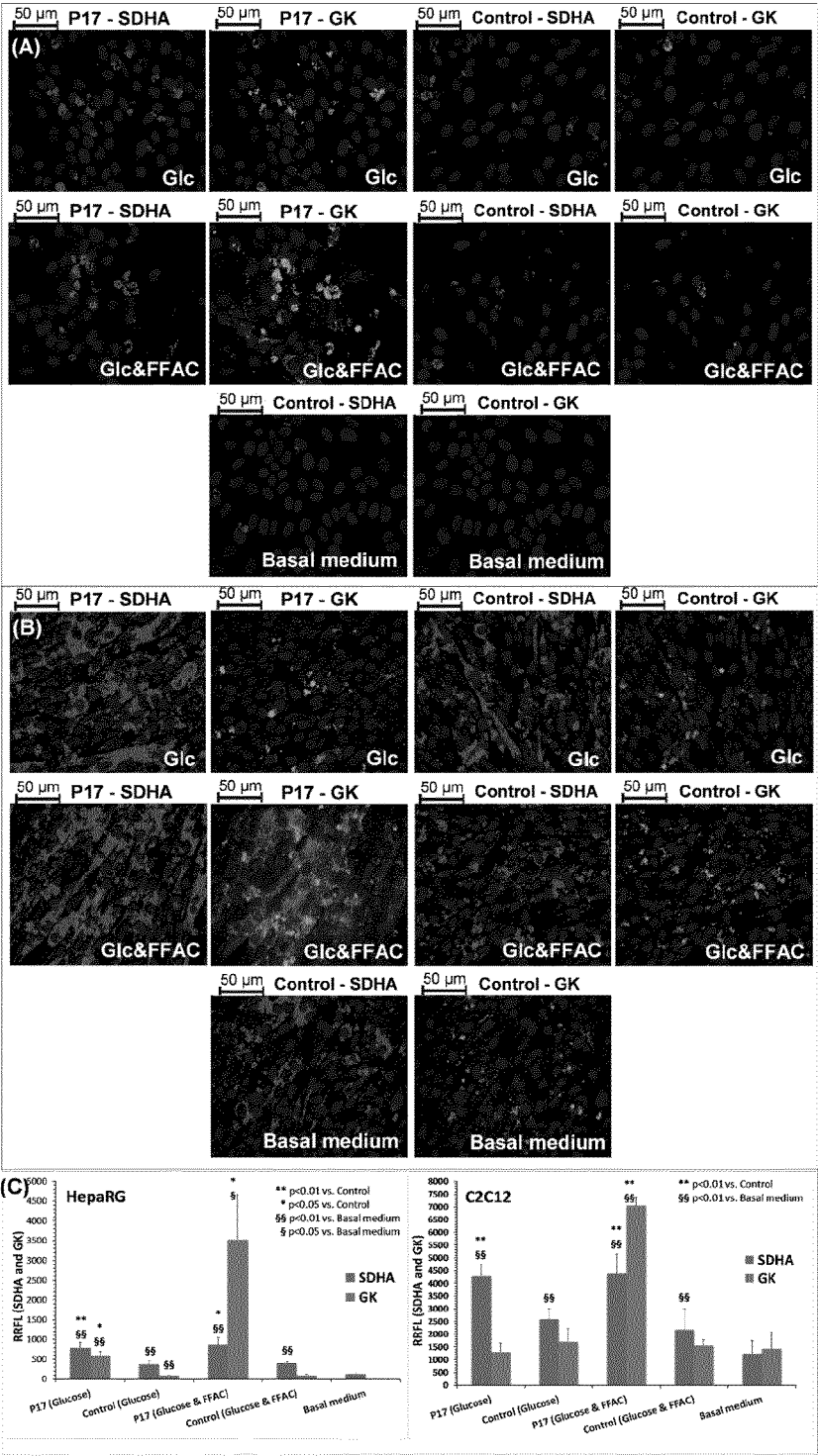


Figure 9

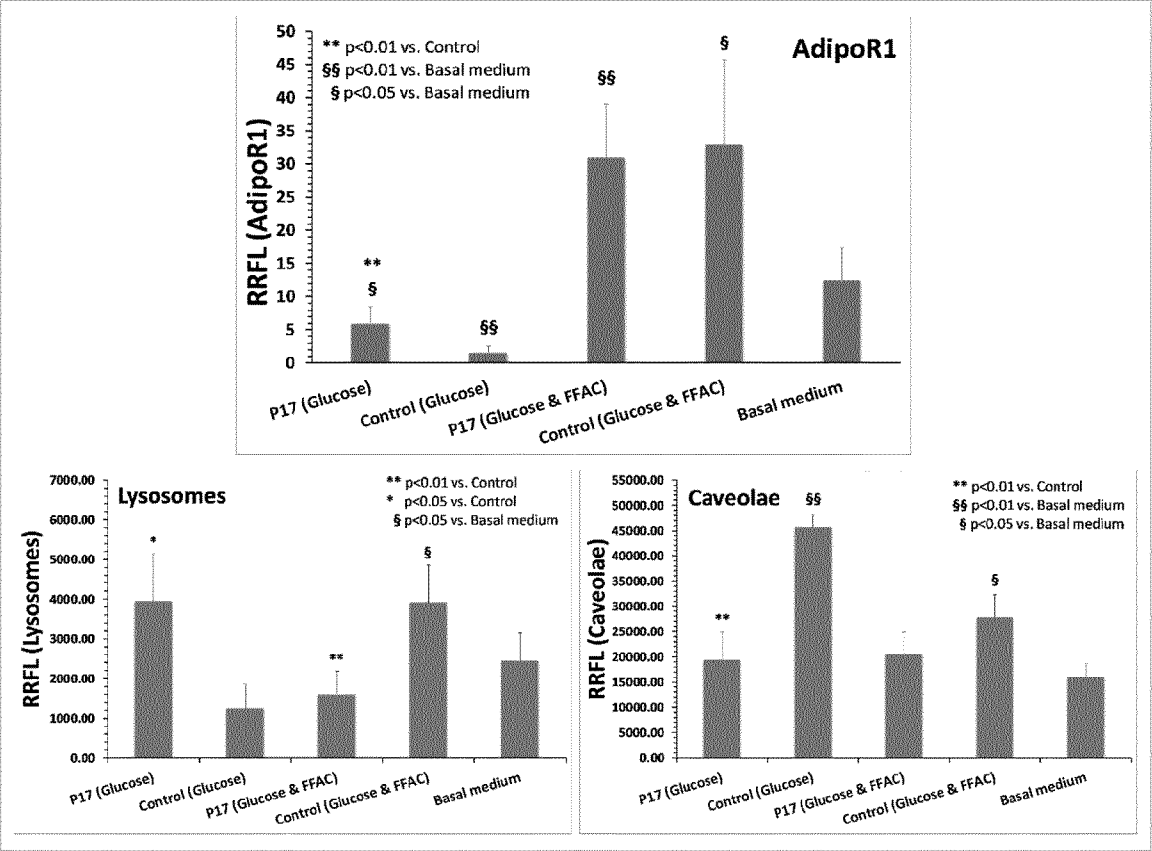


Figure 10

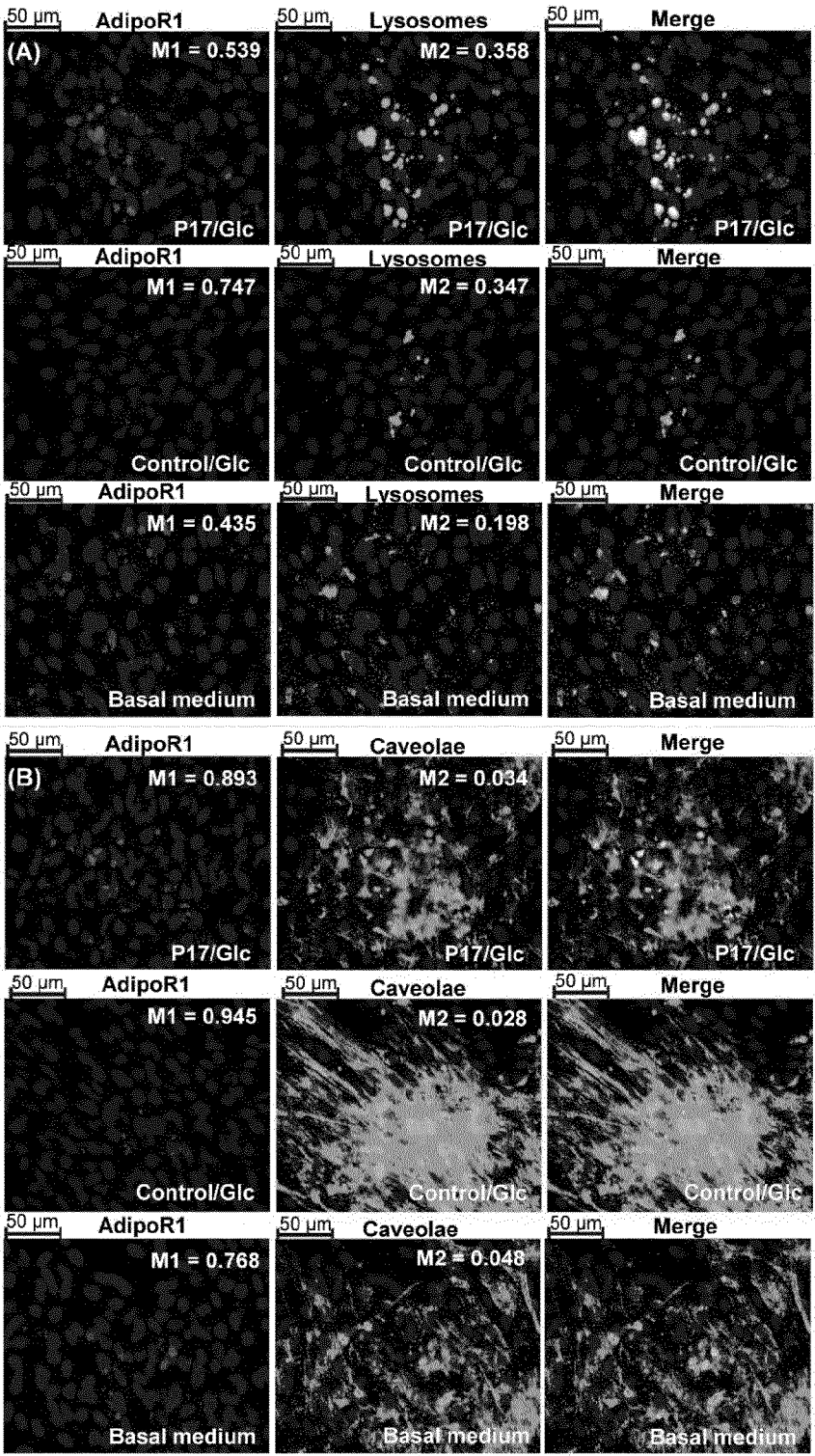


Figure 11

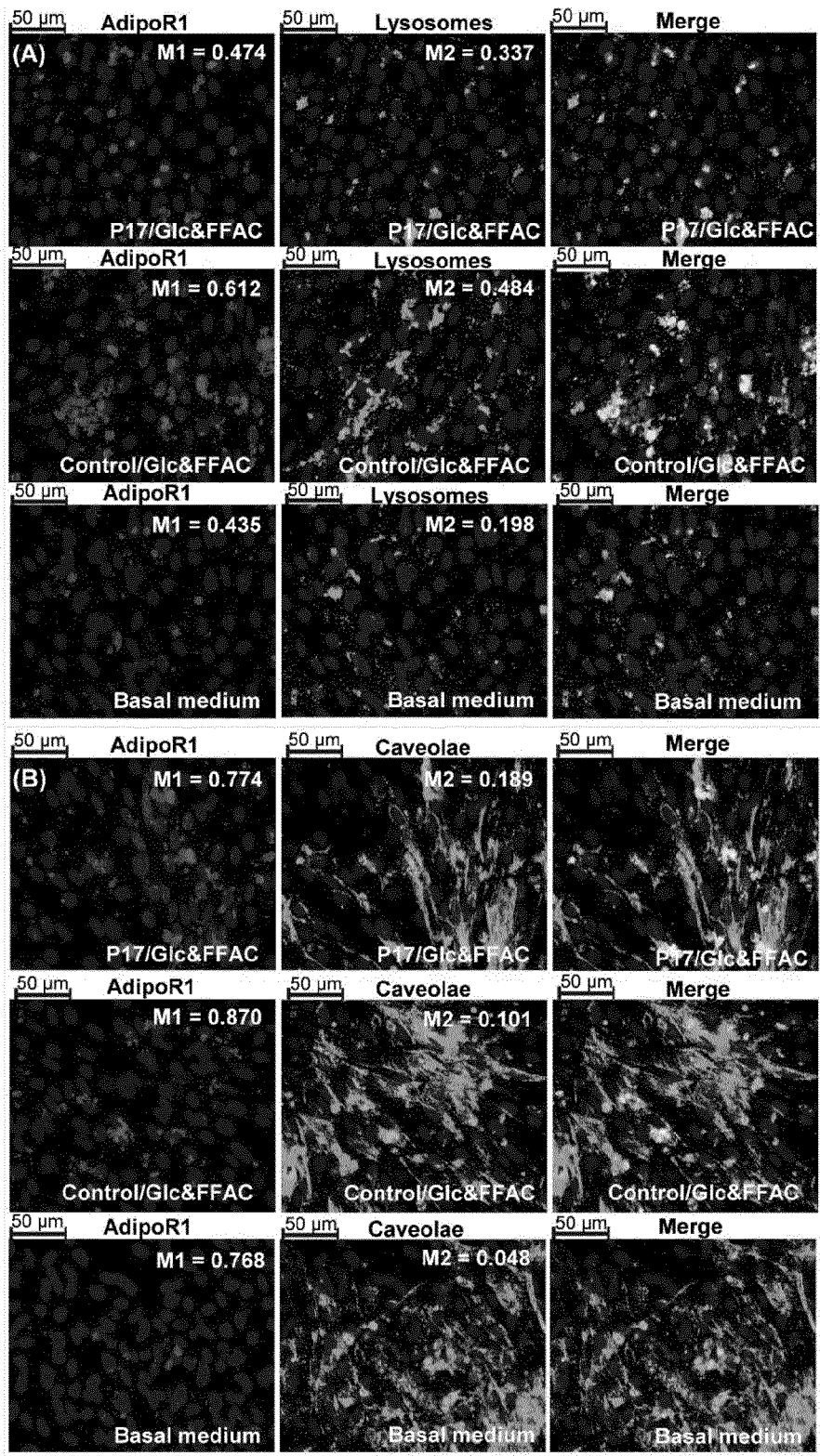


Figure 12

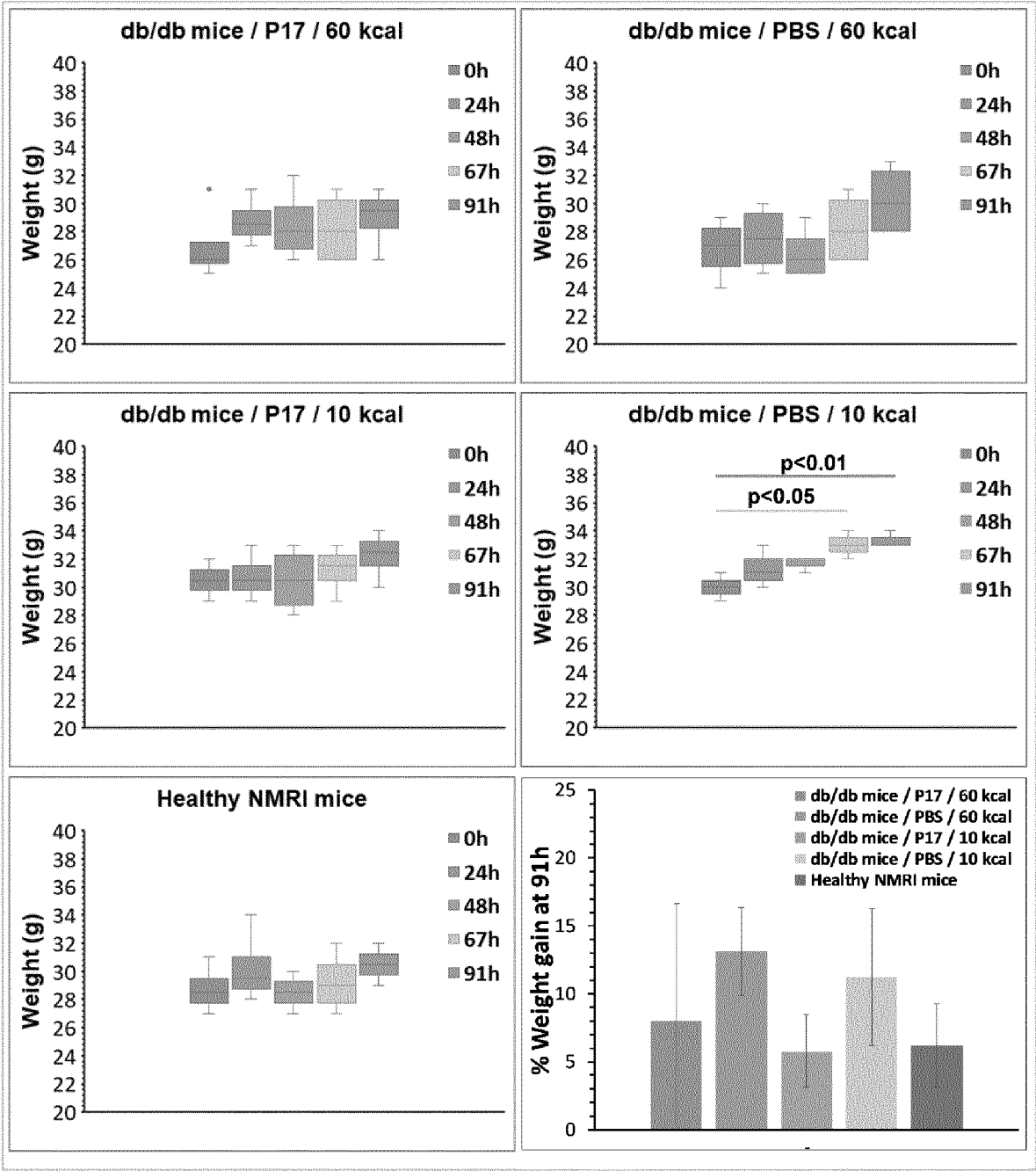


Figure 13

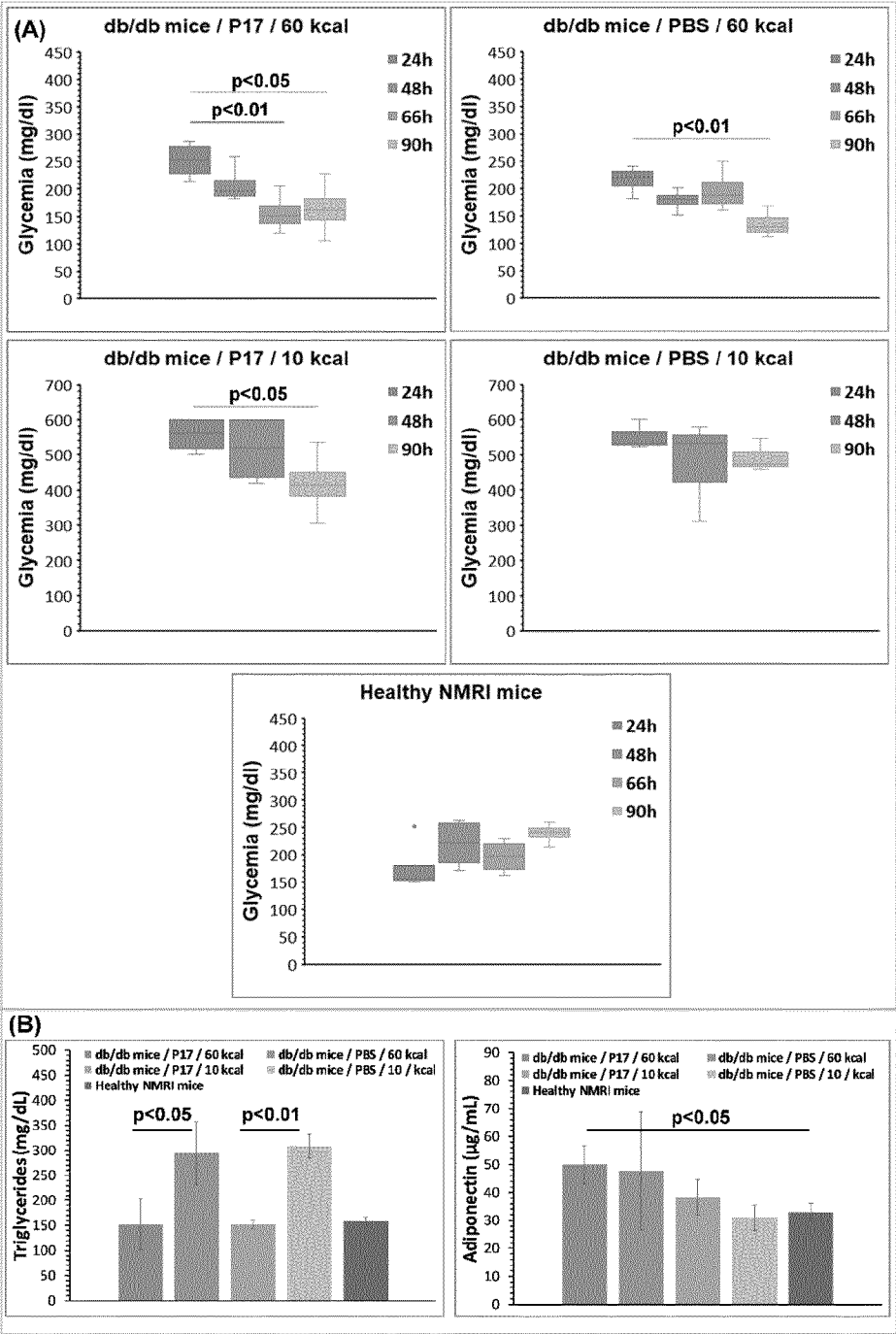


Figure 14

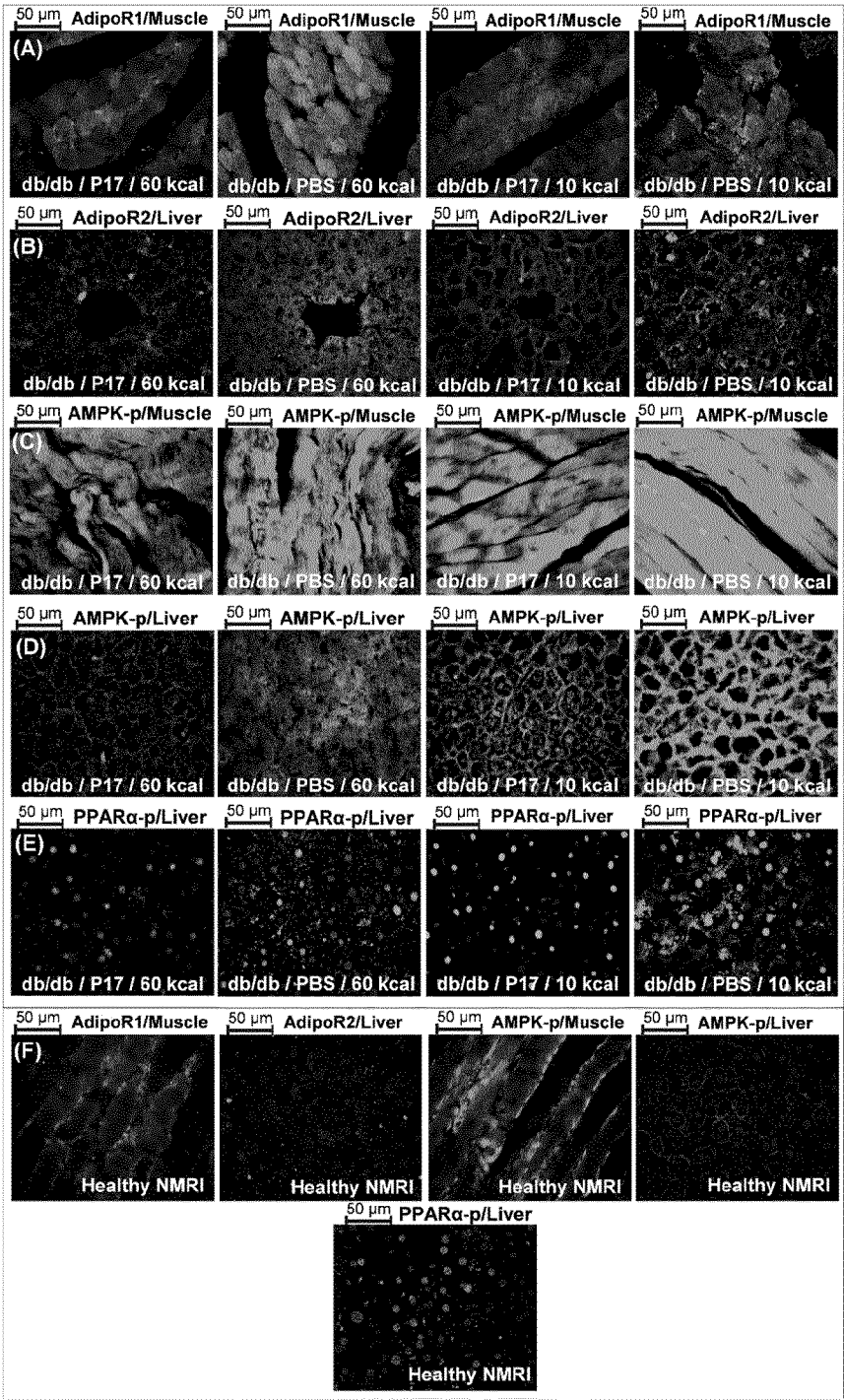


Figure 15

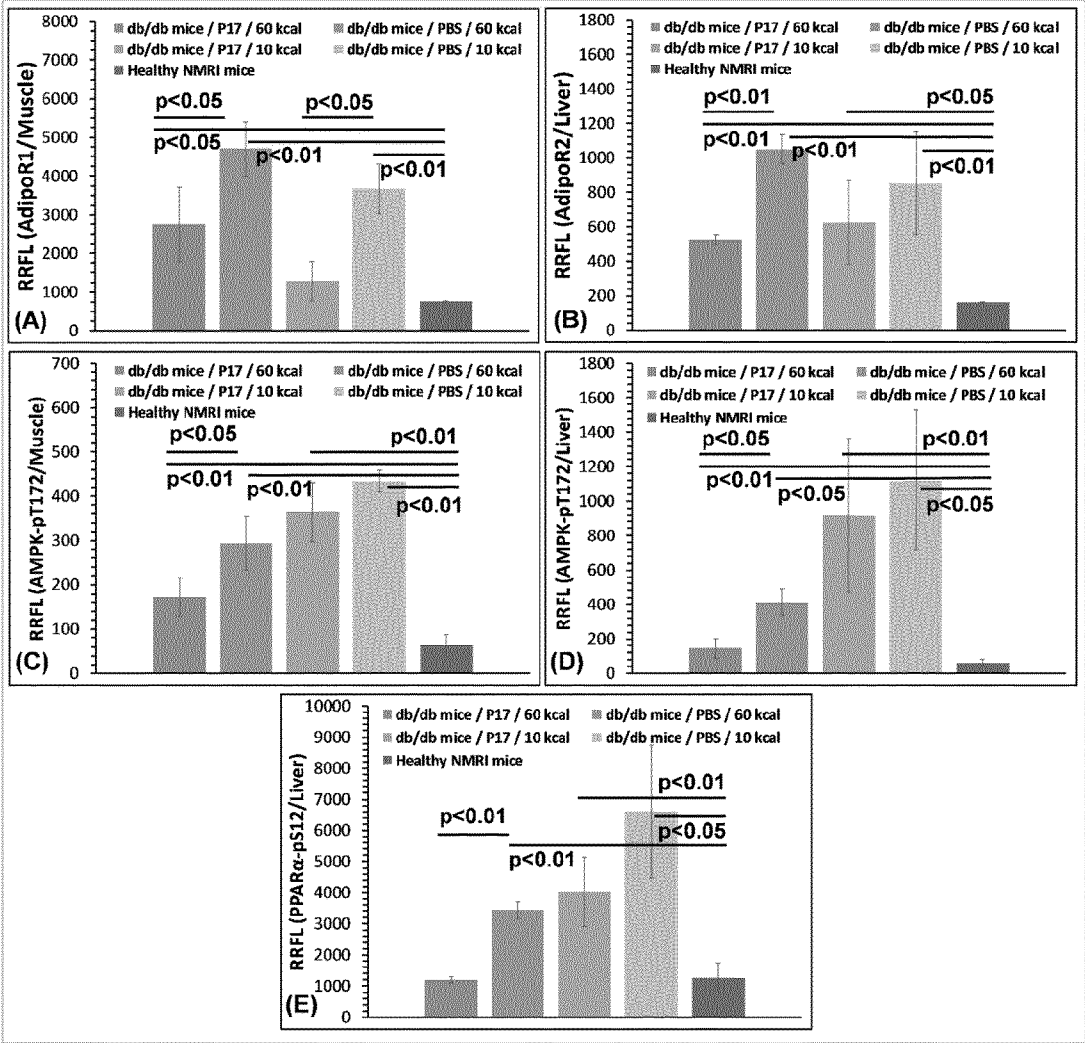


Figure 16

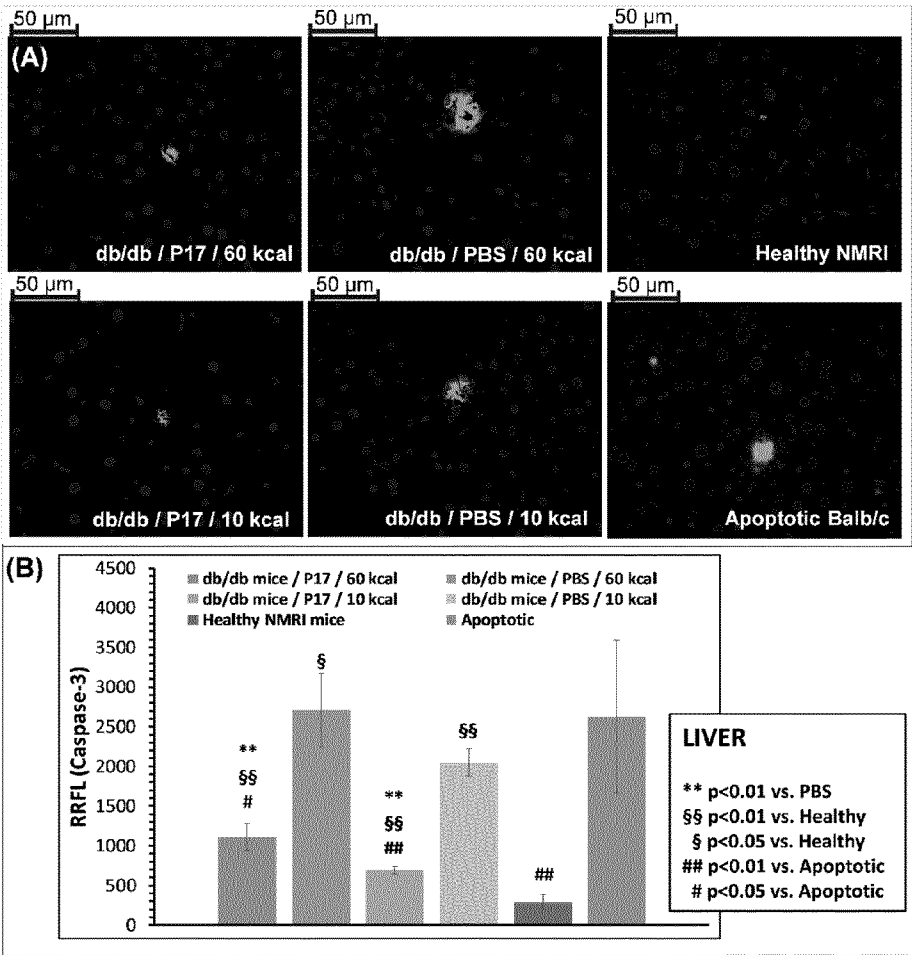


Figure 17

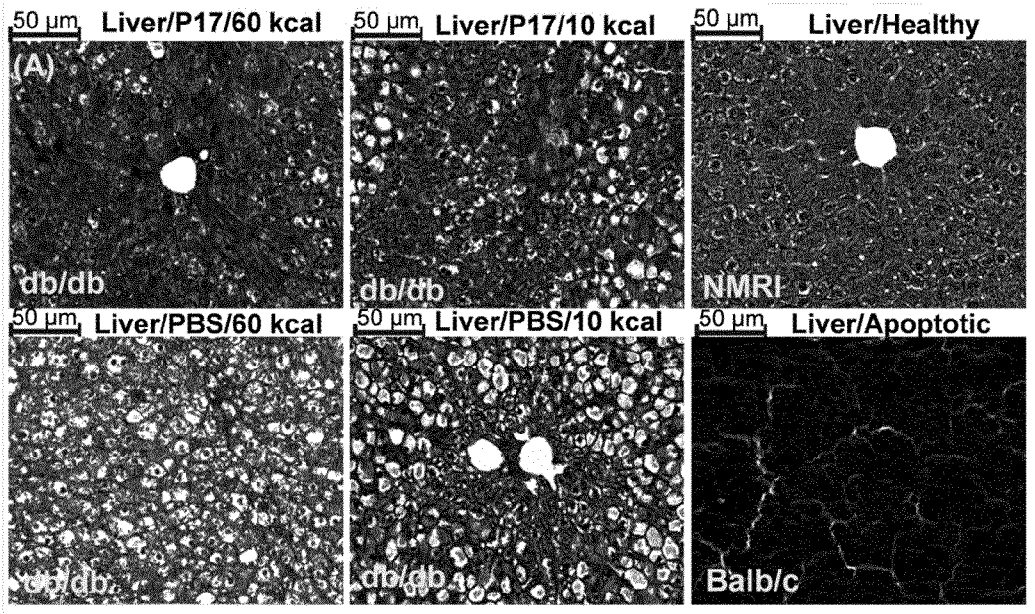


Figure 18

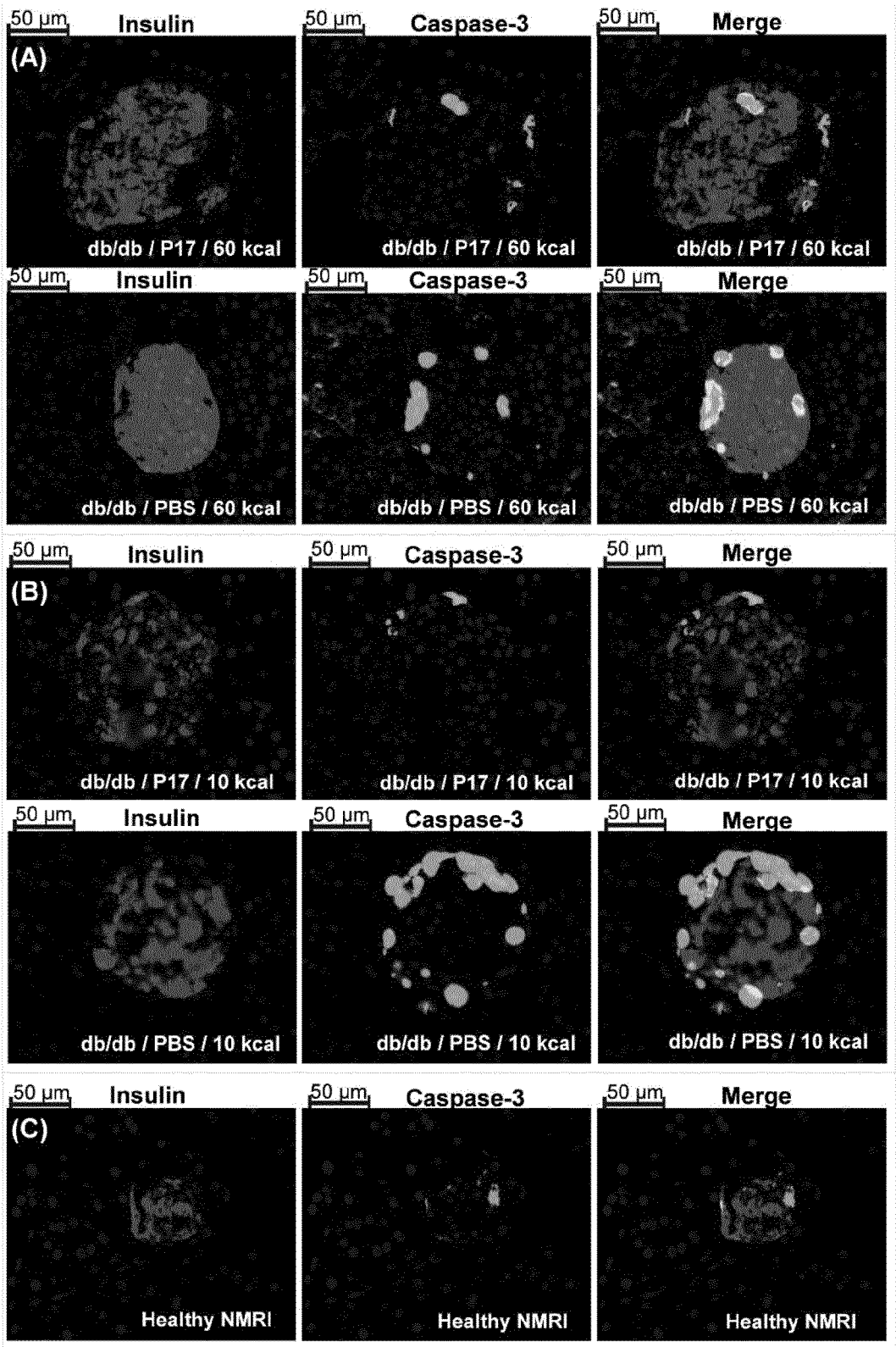


Figure 19

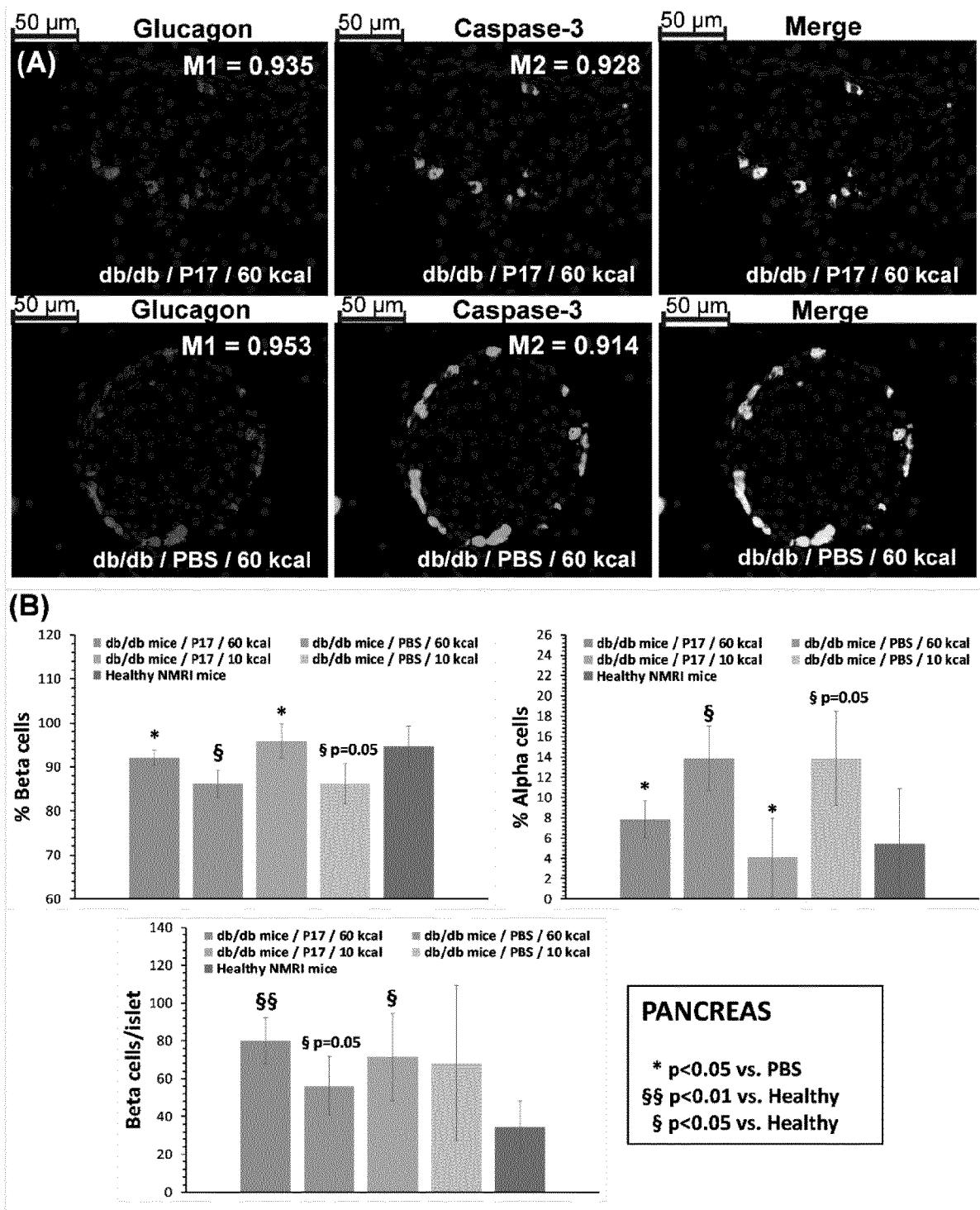


Figure 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/087006

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/10 C07K14/575 C07K14/47
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUNGHWAN KIM ET AL: "Discovery of a novel potent peptide agonist to adiponectin receptor 1", PLOS ONE, vol. 13, no. 6, 18 June 2018 (2018-06-18), page e0199256, XP055587574, DOI: 10.1371/journal.pone.0199256 the whole document	1-17
Y	WO 2012/142142 A2 (UNIV TEMPLE [US]; OTVOS LASZLO [US]; SURMACZ EVA [US]) 18 October 2012 (2012-10-18) claims 1-15 ----- -/--	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 January 2020

Date of mailing of the international search report

20/02/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Marchesini, Patrizia

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/087006

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MIKI OKADA-IWABU ET AL: "A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity", NATURE, vol. 503, no. 7477, 30 October 2013 (2013-10-30), pages 493-499, XP055343097, London ISSN: 0028-0836, DOI: 10.1038/nature12656 abstract -----	1-17
Y	CN 107 290 539 A (UNIV HARBIN MEDICAL) 24 October 2017 (2017-10-24) sequence 12 -----	1-5
Y	WO 2016/150416 A1 (FORSCHUNGSZENTRUM JÜLICH GMBH [DE]) 29 September 2016 (2016-09-29) claims 1-13; sequence 20 -----	1-5,9-15
Y	WO 2009/070350 A1 (SIEMENS HEALTHCARE DIAGNOSTICS INC) 4 June 2009 (2009-06-04) claims 1-90; sequences 1, 7 -----	6-17
Y	MIKI OKADA-IWABU ET AL: "Perspective of Small-Molecule AdipoR Agonist for Type 2 Diabetes and Short Life in Obesity", DIABETES & METABOLISM JOURNAL, vol. 39, no. 5, 1 January 2015 (2015-01-01), page 363, XP055587590, ISSN: 2233-6079, DOI: 10.4093/dmj.2015.39.5.363 the whole document -----	1-17
Y	LASZLO OTVOS JR ET AL: "Design and development of a peptide-based adiponectin receptor agonist for cancer treatment", BMC BIOTECHNOLOGY, BIOMED CENTRAL LTD. LONDON, GB, vol. 11, no. 1, 5 October 2011 (2011-10-05), pages 1-14, XP021110409, ISSN: 1472-6750, DOI: 10.1186/1472-6750-11-90 the whole document -----	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2019/087006

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17

Relates to a peptide capable of binding to adiponectin receptor 1 or 2 having at least 95 % identity to SEQ ID No.: 2-3, 5-12.

1.1. claims: 6-17

Relates to a molecule able to bind to a region having SEQ ID No.: 13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/087006

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012142142 A2	18-10-2012	US 2014057833 A1 WO 2012142142 A2	27-02-2014 18-10-2012

CN 107290539 A	24-10-2017	NONE	

WO 2016150416 A1	29-09-2016	CN 107787326 A DE 102015003503 A1 EP 3271378 A1 JP 2018512410 A US 2018072786 A1 WO 2016150416 A1	09-03-2018 22-09-2016 24-01-2018 17-05-2018 15-03-2018 29-09-2016

WO 2009070350 A1	04-06-2009	CA 2707182 A1 CN 101932728 A EP 2215268 A1 EP 3536336 A1 ES 2699627 T3 US 2009143275 A1 US 2011301089 A1 WO 2009070350 A1	04-06-2009 29-12-2010 11-08-2010 11-09-2019 12-02-2019 04-06-2009 08-12-2011 04-06-2009
