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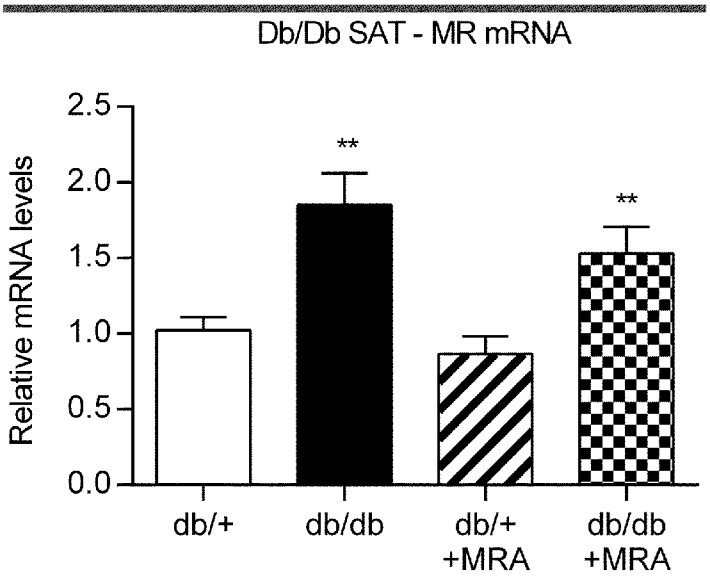

Figure 1A

(57) Abstract: The present invention relates to methods for assessing mineralocorticoid receptor activation in adipose tissue of a subject. In particular, the present invention relates to a method for assessing mineralocorticoid receptor activation in the adipose tissue of a subject comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding of the mineralocorticoid receptor activation in the adipose tissue of the subject when the expression level determined at step i) is higher than the predetermined reference value. The invention also relates to the use of mineralocorticoid receptor antagonists for use in the treatment of metabolic disorders, e.g. metabolic syndrome or obesity.

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METHODS FOR ASSESSING MINERALOCORTICOID RECEPTOR ACTIVATION IN ADIPOSE TISSUE OF A SUBJECT AND TREATING METABOLIC SYNDROME

FIELD OF THE INVENTION:

The present invention relates to methods for assessing mineralocorticoid receptor activation in adipose tissue of a subject.

BACKGROUND OF THE INVENTION:

Metabolic syndrome (MetS) is characterized by a combination of medical disorders strongly associated with the development of cardiovascular diseases and diabetes, two of the major causes of dead according to World Health Organization. MetS associates three or more of the following five clinical variables: blood pressure, abdominal adiposity (given as waist circumference), and fasting values of high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose. Adipose tissue is central in the pathogenesis of MetS: Adipose tissue is not only the energy store of the body, but accordingly to its hormonal functions, it is able to tune food intake, insulin sensitivity and secretion, the vascular function, and the general level of inflammation and oxidative stress status of the body. A better knowledge of molecules either influencing adipose tissue functions or produced by it, is currently a major task in the field.

The hormone Aldosterone is produced by the cortex of the adrenal gland and participates to the control of blood pressure through the regulation of salt and water reabsorption in the renal collecting ducts via activation of its receptor, the mineralcorticoid receptor (MR). MR is a ligand-activated transcription factor whose expression pattern is broader that initially anticipated. In the past decade, evidences were accumulated that MR expression and activation leads to major pathophysiological consequences in organs like heart, vessels, eye and skin, extending the potential therapeutic use of pharmacological MR antagonists. These novel non-classical targets also include adipocytes where its role is still debated.

Experimental and clinical studies have point out that aldosterone is a potential risk factor for diabetes involving mechanisms independent of its effect on blood pressure. A high prevalence (10–50%) of glucose intolerance and/or diabetes has been reported in primary aldosteronism (PA), and these metabolic disturbances were corrected by surgical removal of the aldosterone-producing adenoma. Experimental studies performed in various rodent

models, i.e., db/db, ob/ob or diet-induced obese mice, proposed specific role of aldosterone and/or MR activation. Indeed, mineralocorticoid receptor antagonism (MRA) improves glucose tolerance, decreases insulin resistance, plasma levels of triglycerides and pro-inflammatory cytokines (mice ob/ob, db/db, high fat diet fed). Moreover, expression of the MR itself is not fixed and is increased in adipose tissues in db/db or ob/ob mice (ref). Ex vivo experiments indicate that activation of MR by aldosterone or glucocorticoid influenced adipocyte differentiation and the secretion of adipokines like adiponectin, leptin as well as pro-inflammatory makers. However the specific role of MR in adipose tissue in vivo has not been addressed so far nor the role of increased expression of MR in adipose tissue on the metabolic consequences of MetS.

SUMMARY OF THE INVENTION:

The present invention relates to methods for assessing mineralocorticoid receptor activation in adipose tissue of a subject. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

In one aspect of the invention, the present invention relates to a method for assessing mineralocorticoid receptor activation in the adipose tissue of a subject comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding of the mineralocorticoid receptor activation in the adipose tissue of the subject when the expression level determined at step i) is higher than the predetermined reference value.

As used herein, the term “subject” denotes a mammal, such as a rodent, a feline, a canine, and a primate. Typically, a subject according to the invention is a human.

In some embodiments, the subject suffers from obesity. The term "obesity" refers to a condition characterized by an excess of body fat. The operational definition of obesity is based on the Body Mass Index (BMI), which is calculated as body weight per height in meter squared (kg/m^2). Obesity refers to a condition whereby an otherwise healthy subject has a BMI greater than or equal to 30 kg/m^2, or a condition whereby a subject with at least one co-

morbidity has a BMI greater than or equal to 27 kg/m^2. An "obese subject" is an otherwise healthy subject with a BMI greater than or equal to 30 kg/m^2 or a subject with at least one co-morbidity with a BMI greater than or equal 27 kg/m^2. A "subject at risk of obesity" is an otherwise healthy subject with a BMI of 25 kg/m^2 to less than 30 kg/m^2 or a subject with at least one co-morbidity with a BMI of 25 kg/m^2 to less than 27 kg/m^2. The increased risks associated with obesity may occur at a lower BMI in people of Asian descent. In Asian and Asian-Pacific countries, including Japan, "obesity" refers to a condition whereby a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, has a BMI greater than or equal to 25 kg/m^2. An "obese subject" in these countries refers to a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, with a BMI greater than or equal to 25 kg/m^2. In these countries, a "subject at risk of obesity" is a person with a BMI of greater than 23 kg/m^2 to less than 25 kg/m^2.

In some embodiments, the subject suffers from metabolic syndrome. The term "Metabolic Syndrome", as used herein, is present if a person has three or more of the following symptoms: abdominal obesity, hyperglyceridemia, low HDL cholesterol, high blood pressure, and high fasting plasma glucose. The criteria for these symptoms are defined in the third Report of the National Cholesterol Education Program Expert Panel in Detection, Evaluation and Treatment of High blood Cholesterol in Adults (Ford, ES. et al. 2002).

As used herein, the term "mineralocorticoid receptor" or “MR” has its general meaning in the art and refers to the nuclear receptor subfamily 3, group C, member 2, (NR3C2) that is a receptor with high affinity for mineralocorticoids. The mineralocorticoid receptor is also called aldosterone receptor.

As used herein the term “PTGDS” has its general meaning in the art and refers to prostaglandine D2 synthase.

In some embodiments, the sample is an adipose tissue sample or a blood sample. As use herein the term “blood sample” refers to a whole blood, serum, or plasma sample.

Determination of the expression level of PTGDS can be performed by a variety of techniques. Typically, the determination comprises contacting the sample with selective reagents such as probes, primers or ligands, and thereby detecting the presence, or measuring the amount of nucleic acids or proteins of interest (i.e. PTGDS) originally in the sample.

In a particular embodiment, the expression level may be determined by determining the quantity of mRNA in an adipose tissue sample obtained from the subject.

Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the subject) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis) and/or amplification (e.g., RT-PCR). In a particular embodiment, the expression level PTGDS is determined by RT-PCR, typically quantitative or semi-quantitative RT-PCR, even more typically real-time quantitative or semi-quantitative RT-PCR. Other methods of amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more typically 85% identical and even more typically 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (e. g. avidin/biotin).

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more typically of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly

match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they typically hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the expression level may be determined by determining the quantity of the PTGDS protein in a blood sample obtained from the subject.

In a particular embodiment, the methods of the invention comprise contacting the biological sample with a binding partner capable of selectively interacting with the PTGDS protein present in the blood sample. The binding partner may be an antibody that may be polyclonal or monoclonal, typically monoclonal. In another embodiment, the binding partner may be an aptamer.

Polyclonal antibodies of the invention or a fragment thereof can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred.

Monoclonal antibodies of the invention or a fragment thereof can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally and the EBV-hybridoma technique. Alternatively, techniques described for the production of single chain antibodies (see e.g. U.S. Pat. No. 4,946,778) can be adapted to produce anti-PTGDS, single chain antibodies. Antibodies useful in practicing the present invention also include anti-PTGDS fragments

including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to NGAL or to SERPINA3. For example, phage display of antibodies may be used. In such a method, single-chain Fv (scFv) or Fab fragments are expressed on the surface of a suitable bacteriophage, e. g., M13. Briefly, spleen cells of a suitable host, e. g., mouse, that has been immunized with a protein are removed. The coding regions of the VL and VH chains are obtained from those cells that are producing the desired antibody against the protein. These coding regions are then fused to a terminus of a phage sequence. Once the phage is inserted into a suitable carrier, e. g., bacteria, the phage displays the antibody fragment. Phage display of antibodies may also be provided by combinatorial methods known to those skilled in the art. Antibody fragments displayed by a phage may then be used as part of an immunoassay.

In another embodiment, the binding partner may be an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence.

The binding partners of the invention such as antibodies or aptamers, may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

As used herein, the term "labelled", with regard to the antibody, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labelling of the probe or antibody by reactivity with a detectable substance. An antibody or aptamer of the invention may be labelled with a radioactive

molecule by any method known in the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I123, I124, In111, Re186, Re188.

The aforementioned assays generally involve the binding of the binding partner (ie. Antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

The concentration of PTGDS may be measured by using standard immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize said PTGDS. A biological sample containing or suspected of containing said PTGDS is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Measuring the concentration of the PTGDS protein (with or without immunoassay-based methods) may also include separation of the compounds: centrifugation based on the compound's molecular weight; electrophoresis based on mass and charge; HPLC based on hydrophobicity; size exclusion chromatography based on size; and solid-phase affinity based on the compound's affinity for the particular solid-phase that is used. Once separated, said PTGDS may be identified based on the known "separation profile" e. g., retention time, for that compound and measured using standard techniques.

Alternatively, the separated compounds may be detected and measured by, for example, a mass spectrometer.

A reference value can be relative to a number or value derived from population studies, including without limitation, such subjects having similar body mass index, total cholesterol levels, LDL/HDL levels, systolic or diastolic blood pressure, subjects of the same or similar age range, subjects in the same or similar ethnic group, and subjects having the same severity of cirrhosis. Such predetermined reference values can be derived from statistical analyses and/or risk prediction data of populations obtained from mathematical algorithms and computed indices of metabolic syndrome. In one embodiment of the present invention, the predetermined reference values are derived from the level of PTGDS in a control sample derived from one or more subjects who were not subjected to obesity or metabolic syndrome. Furthermore, retrospective measurement of the level of PTGDS in properly banked historical subject samples may be used in establishing these predetermined reference values. Typically, the levels of PTGDS in a subject having mineralocorticoid receptor activation in the adipose tissue is deemed to be higher than the reference value obtained from the general population or from healthy subjects. In some embodiments, the expression level of PTGDS is deemed to be higher than the predetermined reference value obtained from the general population or from healthy subjects if the ratio of the expression level of PTGDS to that of said predetermined reference value is higher than 1.2, preferably 1.5, even more preferably 2, even more preferably 5, 10 or 20.

The method for assessing mineralocorticoid receptor activation in the adipose tissue according to the invention may find in various applications, In particular, the method of the present invention is particularly suitable for the treatment of subjects suffering from obesity or metabolic syndrome. Even more particularly, mineralocorticoid receptor antagonists have been suggested as beneficial for the treatment of metabolic syndrome. However, up to now, it was not possible to discriminate subjects that could benefit from such a treatment. Administration of a MR antagonist in a subject may be accompanied with serious adverse side effects such as hyperkalemia and therefore it is highly desirable to clearly identify subjects suffering from metabolic syndrome that could benefit of a treatment with a MR antagonist.

In one further aspect, the present invention relates to a method for treating at least one metabolic derangement selected from the group consisting of abdominal obesity, total body

weight gain, hypertension, hypertriglyceridemia, dyslipidemia, glucose intolerance, and insulin resistance in a subject suffering from metabolic syndrome comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist or an inhibitor of mineralocorticoid receptor gene expression when the expression level determined at step i) is higher than the predetermined reference value.

In one further aspect, the present invention relates to a method for simultaneously reducing abdominal obesity, total body weight gain, hypertriglyceridemia, dyslipidemia, glucose intolerance, and insulin resistance associated with metabolic syndrome in a subject in need thereof comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist or an inhibitor of mineralocorticoid receptor gene expression when the expression level determined at step i) is higher than the predetermined reference value.

In one further aspect, the present invention relates to a method for treating metabolic syndrome in a subject in need thereof comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist when the expression level determined at step i) is higher than the predetermined reference value.

As used herein the term "MR antagonist" has its general meaning in the art. The MR antagonistic of a compound may be determined using various methods as described in J, Souque A, Wurtz JM, Moras D, Rafestin-Oblin ME. Mol Endocrinol. 2000 Aug;14(8):1210-21; Fagart J, Seguin C, Pinon GM, Rafestin-Oblin ME. Mol Pharmacol. 2005 May;67(5):1714-22 or Hellal-Levy C, Fagart J, Souque A, Wurtz JM, Moras D, Rafestin-Oblin ME. Mol Endocrinol. 2000 Aug;14(8):1210-21. Typically, the transfection of the human mineralocorticoid receptor in COS cells together with a luciferase-expressing reporter gene allows to measure its transactivation activity in the presence of a candidate compound. In the context of the present invention, mineralocorticoid receptor antagonists are typically

selective for the mineralocorticoid receptor as compared with the related receptors such as androgen receptor, estrogen receptors, glucocorticoid receptor, progesterone receptor, thyroid hormone receptors, peroxisome proliferator-activated receptors, retinoic acid receptor, farnesoid x receptor, pregnane x receptor, liver X receptor, vitamin D receptor, retinoid x receptor and the constitutive androstane receptor. By “selective” it is meant that the affinity of the antagonist for the mineralocorticoid receptor is at least 10-fold, typically 25-fold, more typically 100-fold, still typically 500-fold higher than the affinity for the related receptors. MR antagonists constitute a class o pharmacological compounds that are well known by the skilled artisan.

For example, the mineralocorticoid receptor antagonists according to the invention generally are spirolactone-type steroidal compounds. The term "spirolactone-type" is intended to characterize a structure comprising a lactone moiety attached to a steroid nucleus, typically at the steroid "D" ring, through a spiro bond configuration. A subclass of spirolactone-type mineralocorticoid receptor antagonist compounds consists of epoxy-steroidal mineralocorticoid receptor antagonist compounds such as eplerenone. Another subclass of spirolactone-type antagonist compounds consists of non-epoxy-steroidal mineralocorticoid receptor antagonist compounds such as spironolactone.

The epoxy-steroidal mineralocorticoid receptor antagonist compounds used in the method of the present invention generally have a steroidal nucleus substituted with an epoxy-type moiety. The term "epoxy-type" moiety is intended to embrace any moiety characterized in having an oxygen atom as a bridge between two carbon atoms.

The term "steroidal," as used in the phrase "epoxy-steroidal," denotes a nucleus provided by a cyclopenteno-phenanthrene moiety, having the conventional "A", "B", "C", and "D" rings. The epoxy-type moiety may be attached to the cyclopentenophenanthrene nucleus at any attachable or substitutable positions, that is, fused to one of the rings of the steroidal nucleus or the moiety may be substituted on a ring member of the ring system. The phrase "epoxy-steroidal" is intended to embrace a steroidal nucleus having one or a plurality of epoxy-type moieties attached thereto.

Epoxy-steroidal mineralocorticoid receptor antagonists suitable for use in the present methods include a family of compounds having an epoxy moiety fused to the "C" ring of the

steroidal nucleus. Examples include 20-spiroxane compounds characterized by the presence of a 9α, 11α-substituted epoxy moiety, such as:

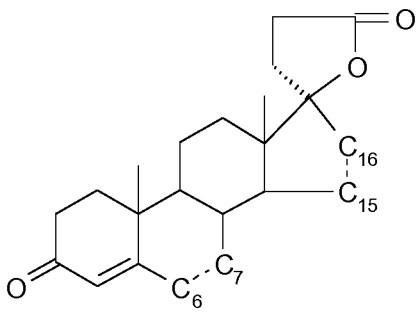
- Pregn-4-ene-7,21-dicarboxylic acid, 9,11-epoxy-17-hydroxy-3-oxo-,γ-lactone, methyl ester, (7α,11α,17β)
- Pregn-4-ene-7,21-dicarboxylic acid, 9,11-epoxy-17-hydroxy-3-oxo-,dimethyl ester, (7α,11α,17β)
- 3′ H-cyclopropa[6,7]pregna-4,6-diene-21- carboxylic acid,9,11-epoxy-6,7-dihydro- 17-hydroxy-3-oxo-, γ -lactone, (6 β ,7 β ,11 α ,17 β)
- Pregn-4-ene-7,21-dicarboxylic acid,9,11- epoxy-17-hydroxy-3-oxo-,7-(1-methylethyl) ester,monopotassium salt, (7α,11α,17β)
- Pregn-4-ene-7,21-dicarboxylic acid,9,11- epoxy-17-hydroxy-3-oxo-,7-methylethyl) ester,monopotassium salt, (7α,11α,17β)
- 3′ H-cyclopropa[6,7]pregna-1,4,6-triene- 21-carboxylic acid,9,11-epoxy-6,7-dihydro- 17-hydroxy-3-oxo-, γ -lactone(6 β ,7 β ,11 α)
- 3′ H-cyclopropa[6,7]pregna-4,6-diene-21- carboxylic acid, 9,11-epoxy-6,7-dihydro-17- hydroxy-3-oxo-, methyl ester, (6 β ,7 β ,11 α ,17 β)
- 3′ H-cyclopropa[6,7]pregna-4,6-diene-21- carboxylic acid, 9,11-epoxy-6,7-dihydro-17- hydroxy-3-oxo-, monopotassium salt, (6 β ,7 β ,11 α ,17 β)
- 3′ H-cyclopropa[6,7]pregna-1,4,6-triene-21- carboxylic acid, 9,11-epoxy-6,7-dihydro-17- hydroxy-3-oxo-, γ -lactone(6 β ,7 β ,11 α ,17 β)
- Pregn-4-ene-7,21-dicarboxylic acid,9,11- epoxy-17-hydroxy-3-oxo-,γ-lactone,ethyl ester,(7α,11α,17β)
- Pregn-4-ene-7,21-dicarboxylic acid,9,11- epoxy-17-hydroxy-3-oxo-,γ-lactone,1- methylethyl ester (7α,11α,17β)

A particular benefit of using epoxy-steroidal mineralocorticoid receptor antagonists, as exemplified by eplerenone, is the high selectivity of this group of mineralocorticoid receptor antagonists for the mineralocorticoid receptor. The superior selectivity of eplerenone results in a reduction in side effects that can be caused by mineralocorticoid receptor antagonists that exhibit non-selective binding to related receptors, such as androgen or progesterone receptors.

These epoxy steroids may be prepared by procedures described in Grob et al., U.S. Pat. No. 4,559,332. Additional processes for the preparation of 9, 11-epoxy steroidal compounds and their salts are disclosed in Ng et al., WO97/21720 and Ng et al., WO98/25948.

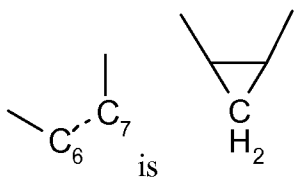
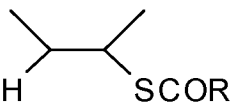
Of particular interest is the compound eplerenone ((Pregn-4-ene-7,21-dicarboxylic acid, 9,11-epoxy-17-hydroxy-3-oxo-,γ-lactone, methyl ester, (7α,11α,17β)) (CAS No. 107724-20-9), also known as epoxymexrenone. Eplerenone is a mineralocorticoid receptor antagonist and has a higher selectivity for mineralocorticoid receptors than does, for example, spironolactone. Selection of eplerenone as the mineralocorticoid receptor antagonist in the present method would be beneficial to reduce certain side-effects such as gynecomastia that occur with use of mineralocorticoid receptor antagonists having less specificity.

Non-epoxy-steroidal mineralocorticoid receptor antagonists suitable for use in the present methods include a family of spirolactone-type compounds defined by Formula I:

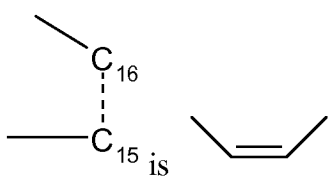
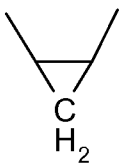
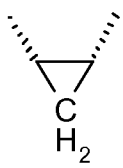


(I)

Wherein:

-  is 

- R is lower alkyl of up to 5 carbon atoms, and

-  is ,  or 

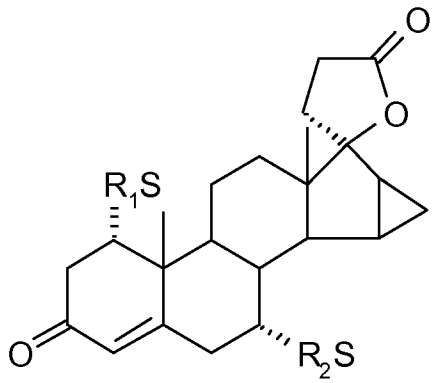
Lower alkyl residues include branched and unbranched groups, for example, methyl, ethyl and n-propyl.

Specific compounds of interest within Formula I are the following:

- 7 α -acetylthio-3-oxo-4,15-androstadiene-[17(β -1′)-spiro-5′]perhydrofuran-2′ -one;
- 3-oxo-7 α -propionylthio-4,15-androstadiene-[17((β -1′)-spiro-5′]perhydrofuran-2′ -one;
- 6 β ,7 β -methylene-3-oxo4,15-androstadiene-[17((β -1′)-spiro-5′]perhydrofuran-2′ -one;
- 15 α ,16 α -methylene-3-oxo-4,7 α -propionylthio-4-androstene[17(β -1′)-spiro-5′]perhydrofuran-2′ -one;
- 6 β ,7 β ,15 α ,16 α -dimethylene-3-oxo-4-androstene[17(β -1′)-spiro-5′]-perhydrofuran-2′ -one;
- 7 α -acetylthio-15 β ,16 β -Methylene-3-oxo-4-androstene-[17(β -1′)-spiro-5′]perhydrofuran-2′ -one;
- 15 β ,16 β -methylene-3-oxo-7 β -propionylthio-4-androstene-[17(β -1′)-spiro-5′]perhydrofuran-2′ -one; and
- 6 β ,7 β ,15 β ,16 β -dimethylene-3-oxo-4-androstene-[17(β -1′)-spiro-5′]perhydrofuran-2′ -one.

Methods to make compounds of Formula I are described in U.S. Pat. No. 4,129,564 to Wiechart et al. issued on 12 Dec. 1978.

Another family of non-epoxy-steroidal compounds of interest is defined by Formula II:



(II)

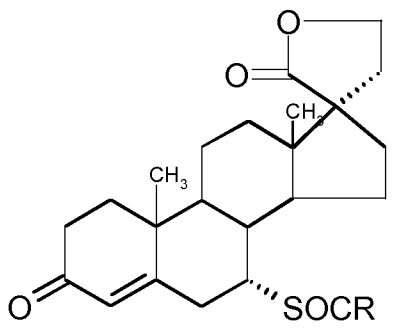
wherein R1 is C1-3-alkyl or C1-3 acyl and R2 is H or C1-3-alkyl.

Specific compounds of interest within Formula II are the following:

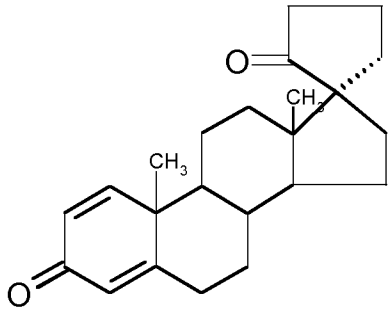
- 1α-acetylthio-15β,16β-methylene-7α-methylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone; and
- 15β,16β-methylene-1α,7α-dimethylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone.

Methods to make the compounds of Formula II are described in U.S. Pat. No. 4,789,668 to Nickisch et al. which issued 6 Dec. 1988.

Yet another family of non-epoxy-steroidal compounds of interest is defined by a structure of Formula III:



or



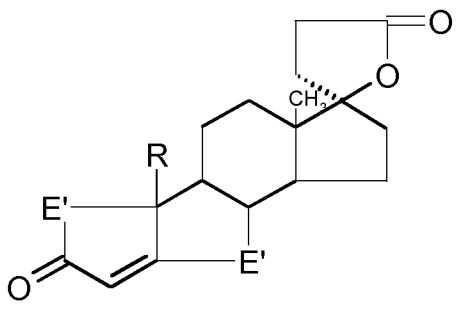
(III)

wherein R is lower alkyl, examples of which include lower alkyl groups of methyl, ethyl, propyl and butyl. Specific compounds of interest include:

- 3β,21-dihydroxy-17α-pregna-5,15-diene-17-carboxylic acid γ-lactone;
- 3β,21-dihydroxy-17α-pregna-5,15-diene-17-carboxylic acid γ-lactone 3-acetate;
- 3β,21-dihydroxy-17α-pregn-5-ene-17-carboxylic acid γ-lactone;
- 3β,21-dihydroxy-17α-pregn-5-ene-17-carboxylic acid γ-lactone 3-acetate;
- 21-hydroxy-3-oxo-17α-pregn-4-ene-17-carboxylic acid γ-lactone;
- 21-hydroxy-3-oxo-17α-pregna-4,6-diene-17-carboxylic acid γ-lactone;
- 21-hydroxy-3-oxo-17α-pregna-1,4-diene-17-carboxylic acid γ-lactone;
- 7α-acylthio-21-hydroxy-3-oxo-17α-pregn-4-ene-17-carboxylic acid γ-lactone; and
- 7α-acetylthio-21-hydroxy-3-oxo-17α-pregn-4-ene-17-carboxylic acid γ-lactone.

Methods to make the compounds of Formula III are described in U.S. Pat. No. 3,257,390 to Patchett which issued 21 Jun. 1966.

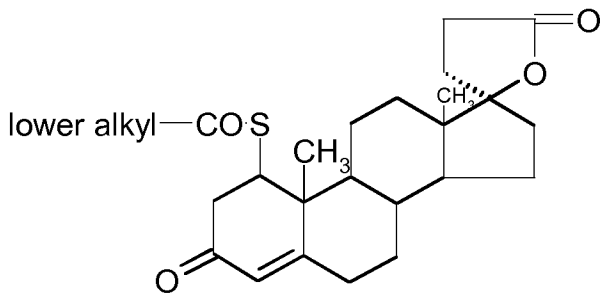
Still another family of non-epoxy-steroidal compounds of interest is represented by Formula IV:



(IV)

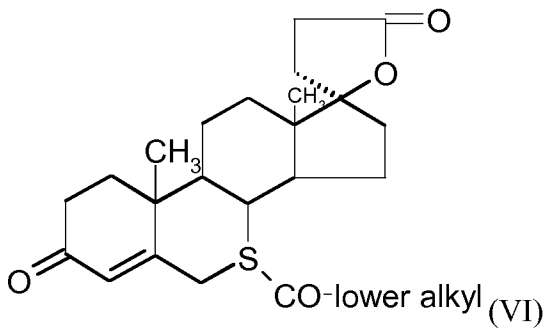
wherein E′ is selected from the group consisting of ethylene, vinylene and (lower alkanoyl)thioethylene radicals, E″ is selected from the group consisting of ethylene, vinylene, (lower alkanoyl)thioethylene and (lower alkanoyl)thiopropylene radicals; R is a methyl radical except when E′ and E″ are ethylene and (lower alkanoyl) thioethylene radicals, respectively, in which case R is selected from the group consisting of hydrogen and methyl radicals; and the selection of E′ and E″ is such that at least one (lower alkanoyl)thio radical is present.

One family of non-epoxy-steroidal compounds within Formula IV is represented by Formula V:



Another compound of Formula V is 1-acetylthio-17α-(2-carboxyethyl)-17β-hydroxy-androst-4-en-3-one lactone.

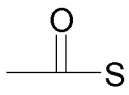
Another family of non-epoxy-steroidal compounds within Formula IV is represented by Formula VI:



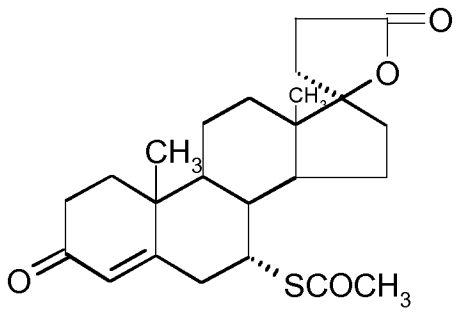
(VI)

Exemplary compounds within Formula VI include the following:

- 7α-acetylthio-17α-(2-carboxyethyl)-17β-hydroxy-androst-4-en-3-one lactone;
- 7β-acetylthio-17α-(2-carboxyethyl)-17β-hydroxy-androst-4-en-3-one lactone;
- 1α,7α-diacetylthio-17α-(2-carboxyethyl)-17β-hydroxy-androsta-4,6-dien-3-one lactone;
- 7α-acetylthio-17αe-(2-carboxyethyl)-17β-hydroxy-androsta-1,4-dien-3-one lactone;
- 7α-acetylthio-17α-(2-carboxyethyl)-17β-hydroxy-19-norandrost-4-en-3-one lactone; and
- 7α-acetylthio-17α-(2-carboxyethyl)-17β-hydroxy-6α-methylandrost-4-en-3-one lactone.

In Formulae IV-VI, the term "alkyl" is intended to embrace linear and branched alkyl radicals containing one to about eight carbons. The term "(lower alkanoyl)thio" embraces radicals of the formula lower alkyl 

Of particular interest is the compound spironolactone (17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone acetate) having the following structure:



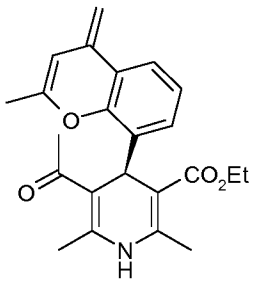
Methods to make compounds of Formulae IV-VI are described in U.S. Pat. No. 3,013,012 to Cella et al. which issued 12 Dec. 1961. Spironolactone is sold by G. D. Searle & Co., Skokie, Ill., under the trademark "ALDACTONE", in tablet dosage form at doses of 25 mg, 50 mg and 100 mg per tablet.

Another family of steroidal mineralocorticoid receptor antagonists is exemplified by drospirenone, (6R-(6α, 7α, 8β, 9α, 10β, 13β, 14α, 15α, 16α, 17β))-1, 3′, 4′, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 21-hexadecahydro-10, 13-dimethylspiro [17H-dicyclopropa(6,7:15,16)cyclopenta(a)phenanthrene-17,2′ (5′ H)-furan)-3,5′ (2H)-dione, CAS registration number 67392-87-4. Methods to make and use drospirenone are described in patent GB 1550568 1979, priority DE 2652761 1976.

Crystalline forms that are easily handled, reproducible in form, easily prepared, stable, and which are non-hygroscopic have been identified for the mineralocorticoid receptor antagonist eplerenone. These include Form H, Form L, various crystalline solvates and amorphous eplerenone. These forms, methods to make these forms, and use of these forms in preparing compositions and medicaments, are disclosed in Barton et al., WO 01/41535 and Barton et al., WO 01/42272 both incorporated herein in their entirety.

Mineralocorticoid receptor antagonists according to the invention may also be non-steroidal. For example, classes of non-steroidal MR antagonists have just begun to emerge over the past few years (Meyers, Marvin J1; Hu, Xiao Expert Opinion on Therapeutic Patents, Volume 17, Number 1, January 2007 , pp. 17-23(7) and Piotrowski DW. Mineralocorticoid Receptor Antagonists for the Treatment of Hypertension and Diabetic NephropathyJ. Med. Chem. 2012, 55, 7957−7966). For instance, dihydropyrymidines have been shown to display MR antagonism (Activation of Mineralocorticoid Receptors by Exogenous Glucocorticoids and the Development of Cardiovascular Inflammatory Responses in Adrenalectomized Rats. Young MJ, Morgan J, Brolin K, Fuller PJ, Funder JW. Endocrinology. 2010 Apr 21). Furthermore, Arhancet el al. disclose other class of non-steroidal MR antagonists (Arhancet GB, Woodard SS, Dietz JD, Garland DJ, Wagner GM, Iyanar K, Collins JT, Blinn JR, Numann RE, Hu X, Huang HC. Stereochemical Requirements for the Mineralocorticoid Receptor Antagonist Activity of Dihydropyridines. J Med Chem. 2010 Apr 21). Other exemplary non-steroidal mineralocorticoid receptor antagonists include but are not limited to those described in US 20090163472 WO2004052847, WO 2008053300 WO2008104306, WO2007025604, WO201264631, WO2008126831, WO2012008435, WO2010104721, WO200985584, WO200978934, WO2008118319, WO200917190, WO200789034, WO2012022121, WO2012022120, WO2011141848 and WO200777961 that are hereby incorporated by reference into the present disclosure.

In a particular embodiment the mineralocorticoid receptor antagonist is selected from the group consisting of: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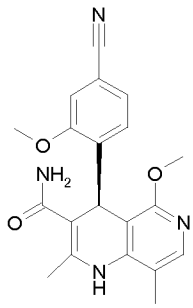
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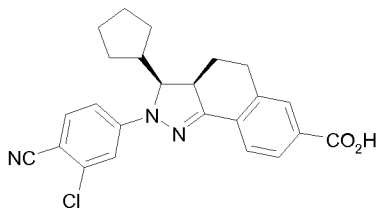
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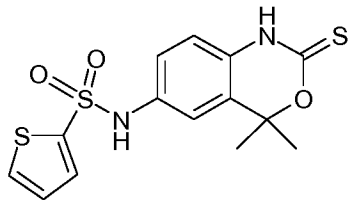
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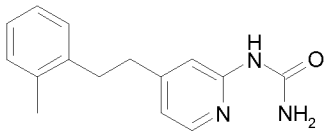
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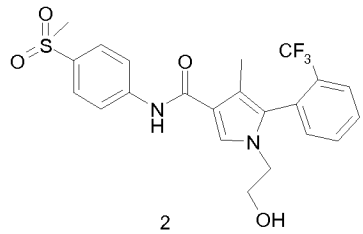
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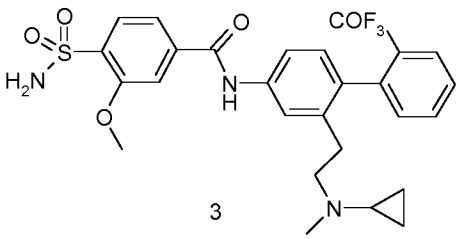
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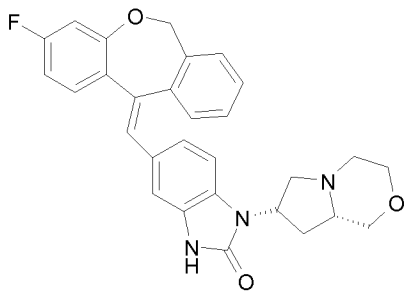
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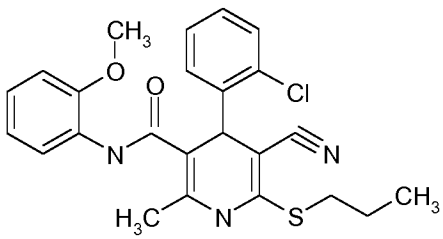
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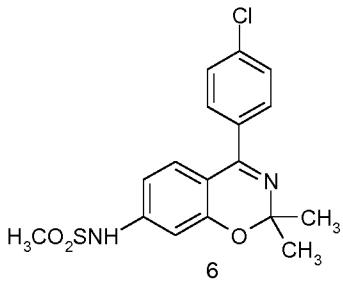
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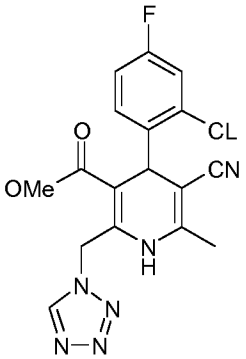
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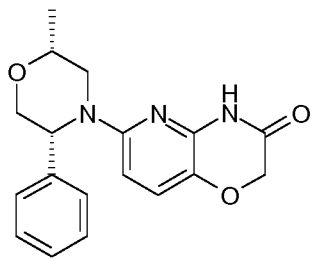
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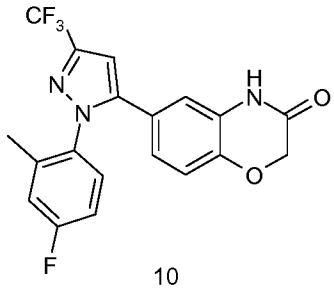


An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. In a preferred embodiment of the invention, said inhibitor of gene expression is a siRNA, an antisense oligonucleotide or a ribozyme. For example, anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of mineralocorticoid receptor mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of mineralocorticoid receptor, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding mineralocorticoid receptor can be synthesized, e.g., by conventional phosphodiester techniques. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732). Small inhibitory RNAs (siRNAs) can also function as inhibitors of expression for use in the present invention. mineralocorticoid receptor gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that mineralocorticoid receptor gene expression is specifically inhibited (i.e. RNA interference or RNAi). Antisense oligonucleotides, siRNAs, shRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid to the cells and typically cells expressing mineralocorticoid receptor. Typically, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral

vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

According to the invention, the active ingredients of the invention (e.g. the MR antagonist or the inhibitor of expression) are administered to the subject in a therapeutically effective amount. By a "therapeutically effective amount" is meant a sufficient amount of the active ingredient for treating or reducing the symptoms at reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination with the active ingredients; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, typically from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Typically the active ingredients of the invention (e.g. the MR antagonist or the inhibitor of expression) is combined with pharmaceutically acceptable excipients, and

optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions.

The term "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

In the pharmaceutical compositions of the present invention, the active ingredients of the invention can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

In one further aspect, the present invention relates to a method to a method for predicting whether a subject suffering from metabolic syndrome is responsive with a treatment with a MR antagonist or an inhibitor of MR gene expression comprising the steps of i) determining the level expression of PTGDS before the treatment, ii) determining the level expression of PTGDS after the treatment, iii) comparing the level determined at step ii) with level determined at step i) and iv) concluding that the subject is responsive to the treatment when the level determined at step ii) is lower than the level determined at step i).

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. MR is overexpressed in adipose tissues from db/db mice. A. Endogenous MR expression is increased by 2-3 fold in SAT and VAT, respectively. B. MRA treatment do not influence MR expression.

Figure 2. Adipocyte aP2-rtTA – Tet-O-transgene mouse model allow the conditional expression of a chosen transgene in adipocytes in vivo. A. Adipocyte-aP2-rtTA – Tet-O-transgene mouse, i.e. Adipo-LacZ and Adipo-MROE, are double transgenic mouse model that results from the crossing of two different mono transgenic mouse model. The first which expresses viral transcription factor rtTA under the control of the adipocyte-specific promoter of aP2 gene, and the second which brings the genetic construct composed of rtTA paired promoter, i.e. Tet-O, and a chosen transgene, i.e., LacZ and hMR, which is intended to be expressed in adipose tissue. In the presence of doxycycline (Dox, 2g/L), rtTA becomes activated and it binds its promoter tet-O, which finally starts transgene expression in aP2 expressing cells. Adipo-lacZ mice conditionally express bacterial β-galactosidase in adipocytes in vivo. Thirty minutes of X-Gal solution exposure of fresh organs taken from Adipo-lacZ mice results immediately in an intense blue staining of only adipose tissues. Three weeks of Dox treatment is sufficient to induce hMR transgene expression in Adipo-MROE mice. **B. and C.** In particularity, qRT-PCR analysis reveals that hMR expression causes a significant increase ($p<0.05$) of total MR mRNA level in both SAT and VAT of Dox treated Adipo-MROE mice.

Figure 3. Adipo-MROE mice displayed weight gain and altered glucose metabolism. Adipo-MROE mice with standard diet presented a significant difference in body mass gain compared to Control-MR littermates (Figure 3A). Increased adipocyte MR expression in VAT induced adipocyte hypertophy (Figure 3B). Interestingly, this increase in adipocyte area is positively correlated with VAT MR expression (Pearson r=0,77, R^2=0.59, $p<0.01$) (Figure 3C). Adipo-MROE mice showed weaker physiological response to insulin as

well as glucose stress charges (Figure 3D and 3E). The analysis of insulin curves during GTT, which take into account the endogenous release of insulin to counteract glucose charge, reveled a higher peak at minute 30 in Adipo-MR mice compared to their Control-MR littermates (Figure 3F).

Figure 4. High-Fat diet exacerbates impairment of insulin response in Adipo-MORE mice. Upon High Fat diet challenge, Adipo-MROE mice showed a higher body mass gain as compared compared to their Control-MR litteramates (Figure 4A). Important impairment in insulin response was clearly impaired in both groups of mice when challenged with 0.05 U/ml of insulin. Adipo-MROE mice displayedvirtually no response to insulin (Figure 4B). Increasing the dose for insulin challenge (0.1 U/ml) allowed insulin response which was impaired in Adipo-MROE mice when compared to Control littermates (Figure 4C). GTT was not statistically different between Control-MR and Adipo-MROE mice (Figure 4D).

Figure 5. The Prostaglandin D2 Synthase (PTGDS) is a novel MR target in adipose tissue. Increased of PTGDS gene expression in adipo-MROE mice: significant PTGDS increase was confirmed in VAT of another series of adipo-MROE mice, as compared to control littermates (Figure 5A). Twenty-four hours aldosterone (Aldo, 10^{-8}M) stimulation in a steroid-free medium increases PTGDS mRNA expression level in differentiated primary cultured adipocytes derived adipo-MROE SAT (Figure 5B) and in differentiated 3T3-L1 adipocytes (Figure 5C). This increase is fully prevented by co-incubation with the MR antagonist spironolactone (MRA, 10^{-6}M) (Figure 5C). Dexamethasone (Dexa, 10^{-8}M) a specific glucocorticoid receptor agonist did not affect PTGDS mRNA levels (Figure 5C). Plasma levels was increased in the plasma of Mice overexpressing MR in adipoMROE mice (Figure 5D) as well as in adipocyte-conditionned medium (ACM) from adipoMROE EVAT (Figure 5 E). PTGDS levels are also increased in the supernatant of 3T3-L1 cells upon treatment with Aldo (Aldo, 10^{-8}M), an effect prevented by MRA (Figure 5F). Of note Dexamethasone (Dexa, 10^{-8}M) has not effect of PTGDS levels (Figure 5F).

Figure 6. The Prostaglandin D2 Synthase (PTGDS) blunted the effects of Aldo in adipocytes. A. Effect of PTGDS antagonism (AT-56) on aldo-induced adipogenesis in 3T3-L1 cells. **B.** Effect of PTGDS antagonism (AT-56) on aldo-induced PPARg2 expression in 3T3-L1 cells. **C.** Effect of PTGDS antagonism (AT-56) on aldo-induced AP2 expression in

3T3-L1 cells. **D.** Effect of PTGDS antagonism (AT-56) on aldo-induced Leptin expression in 3T3-L1 cells. **E.** Effect of PTGDS antagonism (AT-56) on aldo-induced adiponectin expression in 3T3-L1 cells. **F.** PTGDS inhibition also blunted AKT phosphorylation (Ser 473) induced by short-term insulin addition in the presence of Aldo (Aldo, 10^{-8}M), but not in the presence of Dexamethasone (Dexa, 10^{-8}M).

Figure 7. PTGDS is a mineralocorticoid receptor target in adipose tissues from obese db/db mice. In the obese db/db mouse model, PTGDS mRNA levels are increased in VAT of obese mice compared to their control littermates (db/-) (Figure 7A). The increase of PTGDS mRNA levels in VAT and SAT of db/db mice strongly correlates with the increase in MR mRNA levels (Pearson r=0.97, R^2=0.94, p<0.0001) (Figure 7B).

Figure 8. PTGDS is a target of MR activation in human adipose tissue. Aldosterone (Aldo, 10^{-8}M) stimulation increased PTGDS mRNA expression level in the differentiated SW872 human adipocytes (Figure 8A). We observed a 2 fold increase MR expression level is in VAT versus SAT from obese patients (Figure 8B). VAT also showed higher expression level of PTGDS mRNA (2.5 fold) as compared to SAT in these patients (Figure 8C). The correlation analysis between the PTGDS and MR mRNA levels in all analyzed tissues showed a strong positive correlation between these values (Pearson r=0.53, R2=0.40, P<0,0001) (Figure 8D).

Figure 9. PTGDS and MR are also correlated in adipose tissues from obese patients. A. MR expression level in increased in mature adipocyte originating from SART from obese patients (BMI> 30) as compared to leaner patients (BMI < 28). **B.** PTGDS mRNA expression levels followed the same pattern and were increased in mature adipocytes isolated from obese patients. **C.** PTGDS expression was highly correlated to MR expression levels.

EXAMPLE:

Increase MR expression in adipose tissue of db/db mice and Adipo-MROE mice:

Endogenous MR expression has been reported to be increased in various model of metabolic diseases (db/db, ob/ob, HFD). We confirmed this result in SAT and VAT, from BKS.Cg-m+/+Leprdb/J with a 2-3 fold increase in SAT and VAT respectively (Figure 1).

MRA treatment did not influence MR expression (Figure 1). In order to mimic the increased expression of MR expression in adipose tissue we generated a novel mouse model (Adipo-MORE mice) allowing conditional expression of MR in adipose tissue (Figure 2) leading a 3-4 fold increase of total MR expression in in SAT and VAT, respectively (Figure 2.).

Adipo-MROE mice displayed weight gain and altered glucose metabolism.

Adipo-MROE mice with standard diet presented a significant difference in body mass gain compared to Control-MR littermates (Figure 3A). This is associated with an increase in VAT mass (Table 1). Increased adipocyte MR expression in VAT induced adipocyte hypertophy (Figure 3B). Interestingly, this increase in adipocyte area is positively correlated with VAT MR expression (Pearson r=0,77, R^2=0.59, p<0.01) (Figure 3C). In Adipo-MROE mice with standard diet, the fasting plasma insulin concentration and HOMA index, but glycemia is normal, is increased, as compared to littermates (Table 1). Hypertriglyceridemia and hypercholesterolemia is also present (Table 1).

	Low Fat Diet		High Fat Diet	
	Control-MR (n=8)	Adipo-MROE (n=8)	Control-MR (n=18)	Adipo-MROE (n=15)
Body Mass (g)	25.5 ± 0.4	29.3 ± 1.0*	31.9 ± 1.4	37.2 ± 2.0*
VAT/Tibia Ratio	5.0 ± 0.6	10.4 ± 1.8*	17.1 ± 1.8	23.0 ± 2.7
VAT Adipocyte Area (μm^2)	2294 ± 135	3515 ± 325*	3274 ± 237	3688 ± 311
Glycemie (mg/dL)	148 ± 5	160 ± 5	132 ± 6	142 ± 8
Insulin (ng/mL)	1.0 ± 0.1	1.5 ± 0.2*	0.9 ± 0.2	2.35 ± 0.4**
HOMA Index	8.9 ± 0.8	14.5 ± 2.2*	7.6 ± 2.0	20.7 ± 4.4**
Triglycerides (mg/dL)	84.0 ± 5.0	118.0 ± 17*	187 ± 29	181 ± 24
Cholesterol (mg/dL)	86.0 ± 6.0	109.0 ± 6*	184 ± 23	169 ± 8
HDL (mg/dL)	77.0 ± 3.0	83.0 ± 8	120 ± 16	102 ± 12

Table 1. Anthropomorphic and biochemical data relative to Control-MR and Adipo-MROE mice, either submitted to a low fat or a high fat diet. *, p<0,05, **,p<0.01, t test Control-MR vs Adipo-MROE

Glucose metabolism was tested by fasting intraperitoneal insulin tolerance test (ITT,) and glucose tolerance test (GTT). Adipo-MROE mice showed weaker physiological response

to insulin as well as glucose stress charges (Figure 3D and 3E). The analysis of insulin curves during GTT, which take into account the endogenous release of insulin to counteract glucose charge, reveled a higher peak at minute 30 in Adipo-MR mice compared to their Control-MR littermates (Figure 3F). This suggests that Adipo-MR animals need to secrete more insulin to normalize glycaemia. In conclusion increased MR expression in adipocytes led to insulin resistance and increased fat mass, even when mice are fed a standard diet.

High-Fat diet exacerbates impairment of insulin response in Adipo-MORE mice

Upon High Fat diet challenge, Adipo-MROE mice showed a higher body mass gain as compared compared to their Control-MR litteramates (Figure 4A). This difference kept significantly growing for at least 14 weeks with HFD. Important impairment in insulin response was clearly impaired in both groups of mice when challenged with 0.05 U/ml of insulin. Adipo-MROE mice displayedvirtually no response to insulin (Figure 4B). Increasing the dose for insulin challenge (0.1 U/ml) allowed insulin response which was impaired in Adipo-MROE mice when compared to Control littermates (Figure 4C). GTT was not statistically different between Control-MR and Adipo-MROE mice (Figure 4D). In conclusion increased MR expression in adipocyte sensitize increased insulin resistance induced by HFD.

The Prostaglandin D2 Synthase (PTGDS) is a novel MR target in adipose tissue.

In order to identify MR target genes in adipocyte, a microarray analysis comparing total mRNA transcript levels was performed in control-MR mice and in adipo-MORE mice after 3 weeks of MR transgene induction. 101 genes were up-regulated and 246 down-regulated (fold change >1.5, $p<0.05$) in VAT of adipo-MROE mice compared to their control-MR littermates. The genonthology analysis, performed revealed that these genes mainly correspond to genes involved in metabolic pathways of fatty acid synthesis (up-regulation) and immune response, e.g. cytokines receptor (down-regulation).

Among all these genes, we focused on the lipocalin-like prostaglandin D2 synthase (PTGDS) gene due to recent studies showing its involvement in adipose tissue physiology. We first validated increased of PTGDS gene expression in adipo-MROE mice: significant PTGDS increase was confirmed in VAT of another series of adipo-MROE mice, as compared to control littermates (Figure 5A). PTGDS mRNA level is also increased in mature adipocytes isolated from SAT of adipo-MROE mice.

Twenty-four hours aldosterone (Aldo, 10^{-8}M) stimulation in a steroid-free medium increases PTGDS mRNA expression level in in differentiated primary cultured adipocytes derived adipo-MROE SAT (Figure 5B) and in differentiated 3T3-L1 adipocytes (Figure 5C) This increase is fully prevented by co-incubation with the MR antagonist spironolactone (MRA, 10^{-6}M) (Figure 5B-C). Dexamethasone (Dexa, 10^{-8}M) a specific glucocorticoid receptor agonist did not affect PTGDS mRNA levels in 3T3-L1 cells (Figure 5C).

PTGDS is a secreted protein. We therefore analyzed whether PTGDS levels were modulated in the plasma of adipoMROE mice, as well as in the supernatant of 3T3-L1 adipocytes treated with Aldo. Plasma levels was increased in the plasma of Mice overexpressing MR in adipoMROE mice (Figure 5D) as well as in adipocyte-conditionned medium (ACM) from adipoMROE EVAT (Figure 5 E). PTGDS levels are also increased in the supernatant of 3T3-L1 cells upon treatment with Aldo (Aldo, 10^{-8}M), an effect prevented by MRA (Figure 5F). Of note Dexamethasone (Dexa, 10^{-8}M) has not effect of PTGDS levels (Figure 5F).

The Prostaglandin D2 Synthase (PTGDS) blunted the effects of Aldo in adipocytes

AT56 is a competitive antagonist of PTGDS. Co-treatment of 3T3-L1 cells with aldosterone (Aldo, 10^{-8}M) and 10^{-6}M AT56 blunted the effects of Aldo on adipocyte adipogenesis (preventing lipid accumulation (Oil red O, Figure 6 A)), as well as Aldo-induced expression of adipocyte differentiation markers like PPARg2, AP2, leptin and Adiponectin gene expression (Figure 6 B-E). Interestingly, PTGDS inhibition also blunted AKT phosphorylation (Ser 473) induced by short-term insulin addition in the presence of Aldo (Aldo, 10^{-8}M), but not in the presence of Dexamethasone (Dexa, 10^{-8}M) (Figure 6 F).

PTGDS is a mineralocorticoid receptor target in adipose tissues from obese db/db mice.

In the obese db/db mouse model, PTGDS mRNA levels are increased in VAT of obese mice compared to their control littermates (db/-) (Figure 7A) as well in SAT. PTGDS mRNA levels is also increased in mature adipocyte isolated from db/db SAT. The increase of PTGDS mRNA levels in VAT and SAT of db/db mice strongly correlates with the increase in MR mRNA levels (Pearson $r=0.97$, $R^2=0.94$, $p<0.0001$) (Figure 7B). Of note PTGDS mRNA expression levels are no modulated by GR nor correlated to GR expression in VAT and SAT

of db/db mice. Treatment of db/db mice for one month with the MR antagonist eplerenone is effective in blocking PTGDS increase in VAT and SAT (Figure 7A).

PTGDS is a target of MR activation in human adipose tissue.

Aldosterone (Aldo, 10^{-8}M) stimulation increased PTGDS mRNA expression level in the differentiated SW872 human adipocytes (Figure 8A). This increase is prevented by coincubation of aldosterone with the MR antagonist spironolactone (MRA, 10^{-6}M) (Figure 8 A).

We observed a 2 fold increase MR expression level is in VAT versus SAT from obese patients (Figure 8B). VAT also showed higher expression level of PTGDS mRNA (2.5 fold) as compared to SAT in these patients (Figure 8C). Interestingly, the correlation analysis between the PTGDS and MR mRNA levels in all analyzed tissues showed a strong positive correlation between these values (Pearson r=0.53, R^2=0.40, P<0,0001) (Figure 8D).

We next analyzed whether PTGDS and MR were also correlated in adipose tissues from obese patients. MR expression level in increased in mature adipocyte originating from SART from obese patients (BMI> 30) as compared to leaner patients (BMI < 28) (Figure 9A). PTGDS mRNA expression levels followed the same pattern and were increased in mature adipocytes isolated from obese patients (Figure 9B). Interesting PTGDS expression was highly correlated to MR expression levels (Figure 9C).

Discussion:

Our results indicate, for the first time that targeted increase of MR in adipose tissue only results in Metabolic syndrome (MetS), with abdominal obesity and total body weight gain, glucose intolerance and insulin resistance as well as dyslipidemia. Moreover we identified PTGDS as a novel MR target in the adipocyte, both in rodent and in human adipose tissue, unraveling PTGDS as a novel mediator of MR activation in MetS.

MR and metabolic syndrome: potential benefit of MR antagonists

In genetically obese db/db mice, it was reported that pharmacological treatment with the selective MR antagonist eplerenone for 16 weeks reversed all obesity-related changes in adipose tissue gene expression (increased expression of PAI-1, leptin and pro-inflammatory cytokines TNF-a and MCP-1 with a concomitant reduction of PPARc and adiponectin). Short-term eplerenone administration (3 weeks) in db/db and ob/ob mice also showed

improved insulin sensitivity while MR antagonism restored the dysregulation in adipose gene expression in both models. These studies indicated that MR activation is involved in dysregulation of adipose tissue endocrine function that is associated with genetic obesity and HFD challenge. However such a pharmacological approach could not discriminate between direct consequences of MR blockade in the adipose tissue and secondary effects of global MR antagonism in other important targets involved in metabolic syndrome such as vasculature pancreas, liver or muscle where MR has been reported to be expressed and/or involved in insulin secretion and sensitivity. The genetic targeted approach used in the present study allowed to discriminate between these two possibilities: a moderate increase of PTGDS is correlated with the MR expression. We and others have observed in obese mouse models (dbdb and/or ob/ob), induced profound changes in adipocyte biology leading to glucose intolerance, insulin resistance, increased HOMA index and dyslipidemia. Increased MR expression in adipocyte furthermore sensitized the mouse to high fat diet, worsening the consequences of such diet challenge on glucose intolerance and insulin resistance. Blood pressure was not affected but we cannot exclude that a prolonged survey would lead to a different conclusion. Of note, MetS in dbdb or obob mice or after HFD-challenge is not accompanied by increased blood pressure. Targeted increase in MR expression in adipocytes also led to overweight as compared to control mice, both in basal condition or after HFD challenge. This is associated with an increased weight of the fat depots, including perivisceral adipose tissues, without adipocyte hypertrophy, in accordance with ex vivo data indicating that MR activation is proadipogenic.

Molecular targets of MR in adipocytes

Chronic exposure to aldosterone in 3T3-L1 and 3T3-F442A white preadipocytes induced major changes in morphological, biochemical and molecular markers of adipose differentiation. Use of specific MR antagonists or genetic tools such as siRNA directed against MR showed that MR activation and not GR activation was involved in the aldosterone-mediated induction of cell proliferation and induction of the adipogenic transcriptional machinery, up-regulating C/EBPa and PPARc gene expression, with subsequent increase in intracellular triglyceride accumulation and adipokines expression. For instance, it was shown that pharmacological blockade of the MR inhibits critical pathways controlling adipose differentiation in 3T3-L1 cells, via inhibition of clonal expansion, a phase of active DNA synthesis following cell confluence, and by interfering with the transcriptional control of adipose conversion through inhibition of PPARc expression. Importantly, they

showed that MR antagonism is able to block adipocyte differentiation ex vivo also in human primary preadipocytes from different fat depots, giving the MR a relevant role in the pathophysiology of adipose dysfunction in humans. While pioneering studies suggested that aldosterone promotes adipogenesis, more recent studies demonstrated a critical role for the MR induced both by mineralocorticoids and glucocorticoids. For instance it was elegantly demonstrated that the endogenous rodent glucocorticoid corticosterone activates MR in adipocytes. Whether aldosterone and/or glucocorticoids are the natural MR ligands in adipocytes in vivo is an open question. Due to the very low expression level of the selectivity enzyme 11HSD2 and the excess of glucocorticoids over aldosterone in the plasma, it is tempting to propose that glucocorticoids are the endogenous ligands of adipocyte MR. However, the reported association of primary aldosteronism and metabolic alterations also suggest that aldosterone could also acts on its receptor in adipose tissue. Since aldosterone is highly lipophylic and less prone to plasma protein binding, it may accumulate in adipocytes and activates the MR. Moreover, it has been recently demonstrated that adipocytes produce aldosterone, leading to local paracrine MR activation in adipose tissue. In the present study we used aldo in the ex vivo experiments in order to avoid confounding effects of GR activation when using glucocorticoids.

Besides candidate genes obviously involved in adipocyte biology and metabolic alteration such as adiponectin, leptin, PParG, etc, little is known about the specific molecular targets of MR in adipocytes. This novel mouse model gave us the opportunity to address this point in vivo using a non-biased approach. A transcriptomic analysis was performed on perivisceral adipose tissue after 3week induction of MR overexpression without a priori about the ligand that is activating the adipocyte MR in vivo. Among the genes whose expression is dysregulated, we focused our attention on PTGDS, a gene that has been consistently involved in metabolic alterations but has not been identified previously as a MR target. We confirmed that by several approaches that PTGDS expression is up-regulated upon MR activation in differentiated 3T3 cells, in transgenic adipocytes derived from the vascular fraction of adipo-MR fat and in db/db mice. Interestingly in all our experimental conditions, there is a strong correlation between MR expression levels and PTGDS expression. Moreover a non-classical GRE is present in the promoter, which appears functionally activated by MR. Pharmacological MR antagonism, both ex vivo in cultured adipocytes and more importantly, in vivo in dbdb mice blunted PTGDS expression. Finally PTGDS is also an MR target in human adipocytes, both in culture and in human adipose tissue, as evidenced by the correlation between MR expression and PTGDS expression in human fat depot.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS:

1. A method for assessing mineralocorticoid receptor activation in the adipose tissue of a subject comprising the steps of i) determining the expression level of prostaglandine D2 synthase (PTGDS) in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding of the mineralocorticoid receptor activation in the adipose tissue of the subject when the expression level determined at step i) is higher than the predetermined reference value.

2. The method of claim 1 wherein the subject suffers from obesity.

3. The method of claim 1 wherein the subject suffers from metabolic syndrome.

4. The method of claim 1 wherein the sample is an adipose tissue sample

5. The method of claim 1 wherein the sample is a blood sample.

6. The method of claim 4 wherein the expression level of PTGDS is determined by determining the quantity of mRNA in the adipose tissue sample obtained from the subject.

7. The method of claim 5 which comprises contacting the blood sample with a binding partner capable of selectively interacting with the PTGDS protein present in the blood sample.

8. The method of claim 7 wherein the binding partner is an antibody that may be polyclonal or monoclonal, typically monoclonal.

9. The method of claim 7 wherein the binding partner is an aptamer.

10. The method of claim 1 wherein the predetermined reference value is derived from the level of PTGDS in a control sample derived from one or more subjects who were not subjected to obesity or metabolic syndrome.

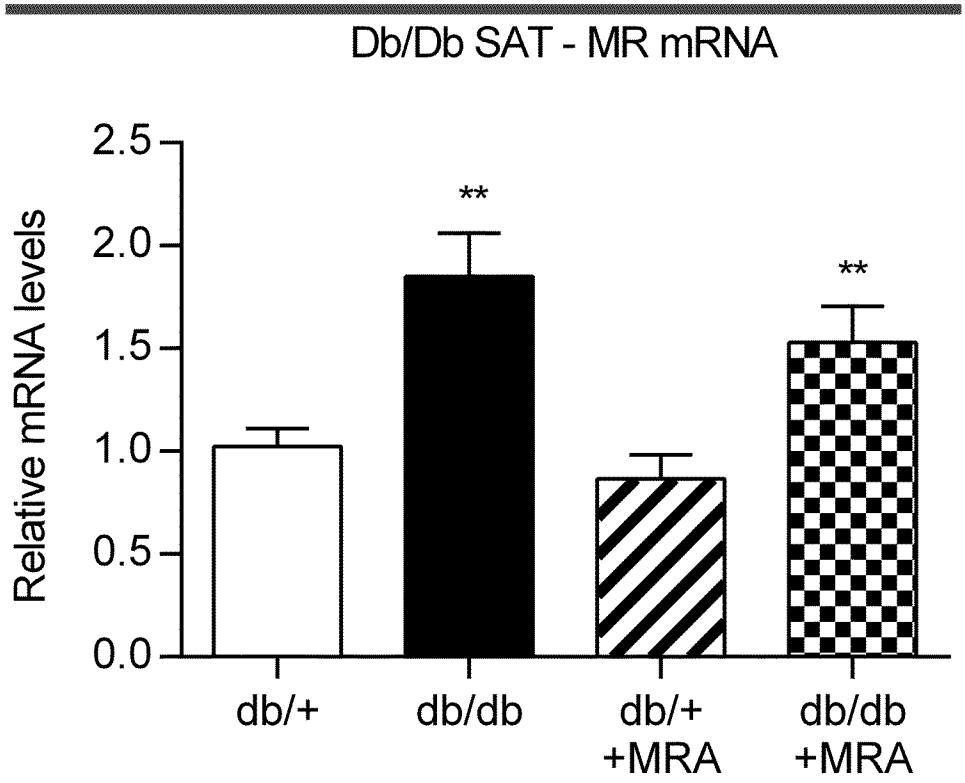
11. A method for treating at least one metabolic derangement selected from the group consisting of abdominal obesity, total body weight gain, hypertension,

hypertriglyceridemia, dyslipidemia, glucose intolerance, and insulin resistance in a subject suffering from metabolic syndrome comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist or an inhibitor of mineralocorticoid receptor gene expression when the expression level determined at step i) is higher than the predetermined reference value.

12. A method for simultaneously reducing abdominal obesity, total body weight gain, hypertriglyceridemia, dyslipidemia, glucose intolerance, and insulin resistance associated with metabolic syndrome in a subject in need thereof comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist or an inhibitor of mineralocorticoid receptor gene expression when the expression level determined at step i) is higher than the predetermined reference value.

13. A method for treating metabolic syndrome in a subject in need thereof comprising the steps of i) determining the expression level of PTGDS in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of a mineralocorticoid receptor antagonist when the expression level determined at step i) is higher than the predetermined reference value.

14. A method to a method for predicting whether a subject suffering from metabolic syndrome is responsive with a treatment with a MR antagonist or an inhibitor of MR gene expression comprising the steps of i) determining the level expression of PTGDS before the treatment, ii) determining the level expression of PTGDS after the treatment, iii) comparing the level determined at step ii) with level determined at step i) and iv) concluding that the subject is responsive to the treatment when the level determined at step ii) is lower than the level determined at step i).



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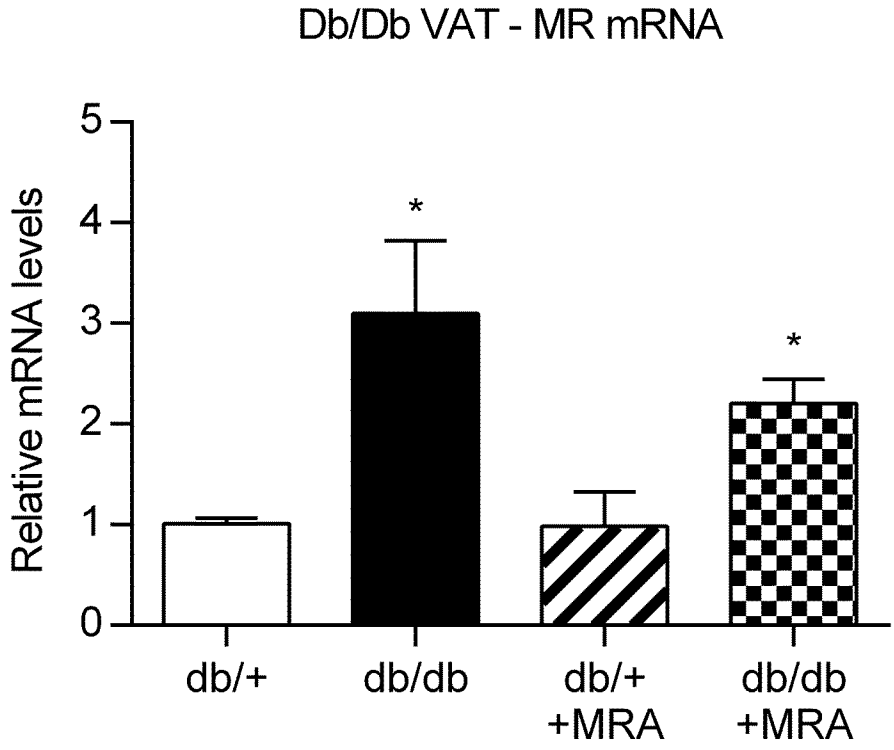

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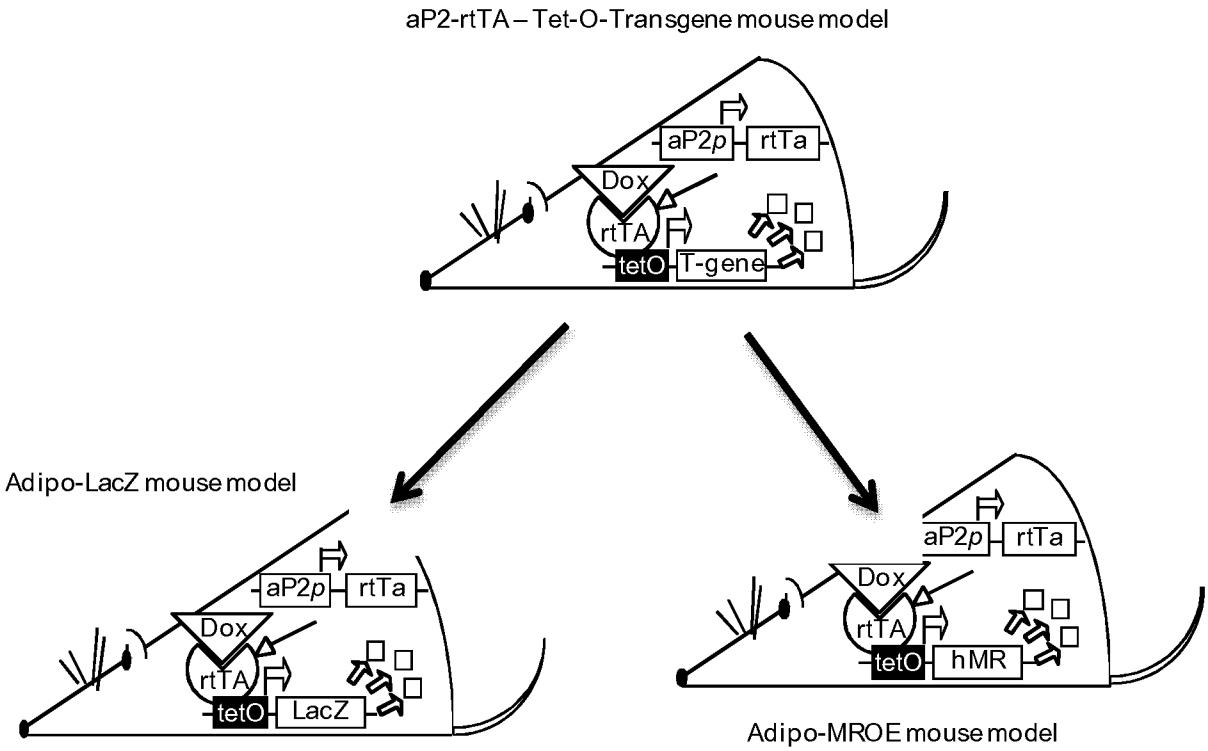
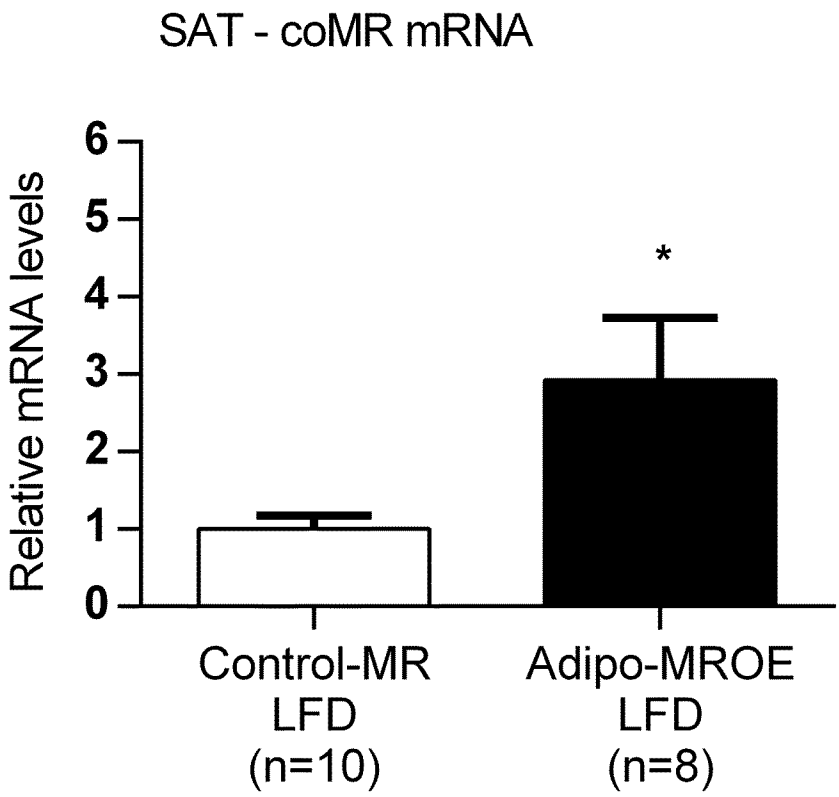


Figure 2A



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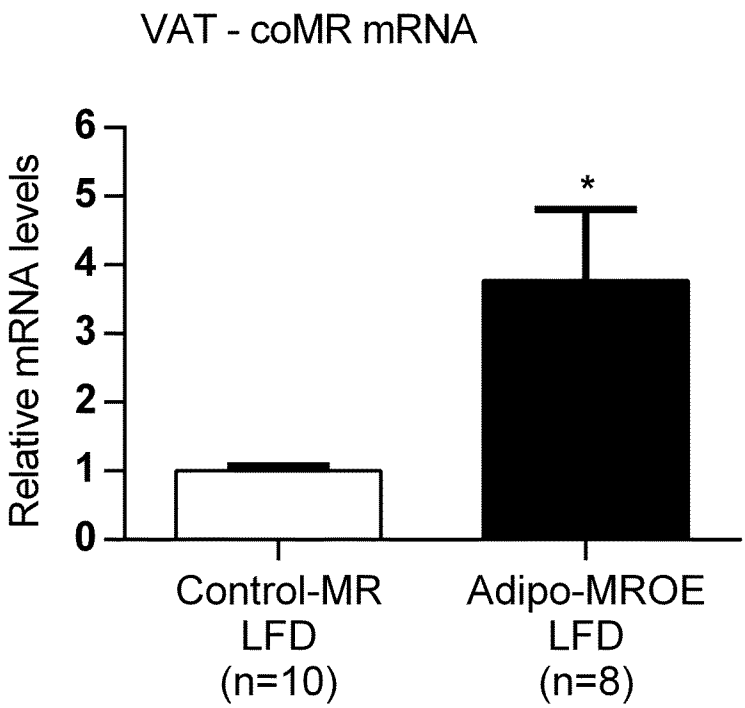


Figure 2C

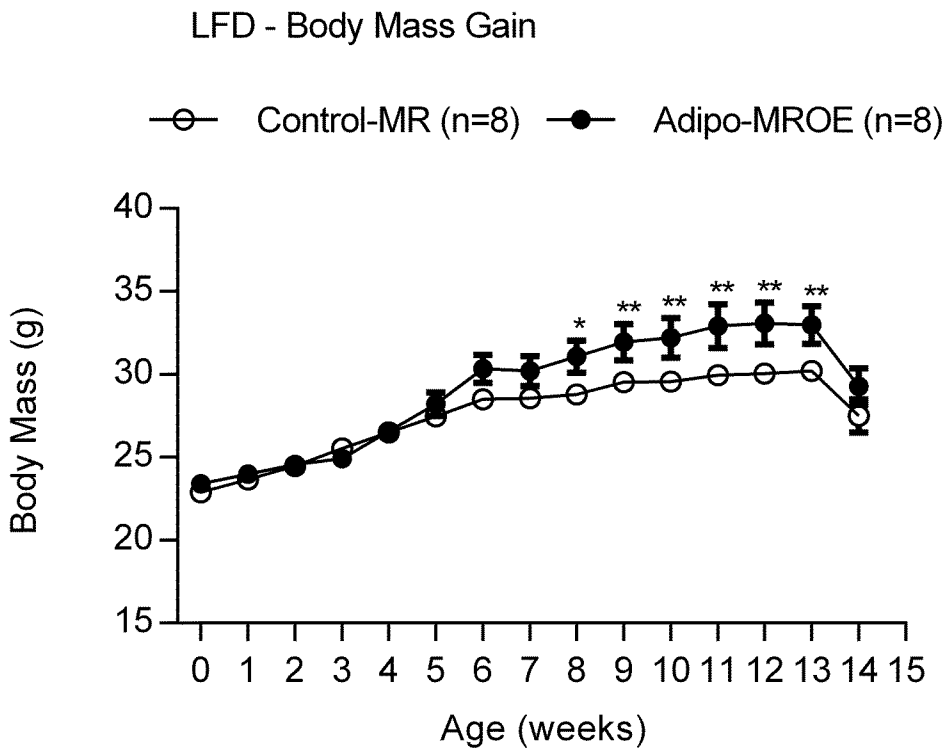
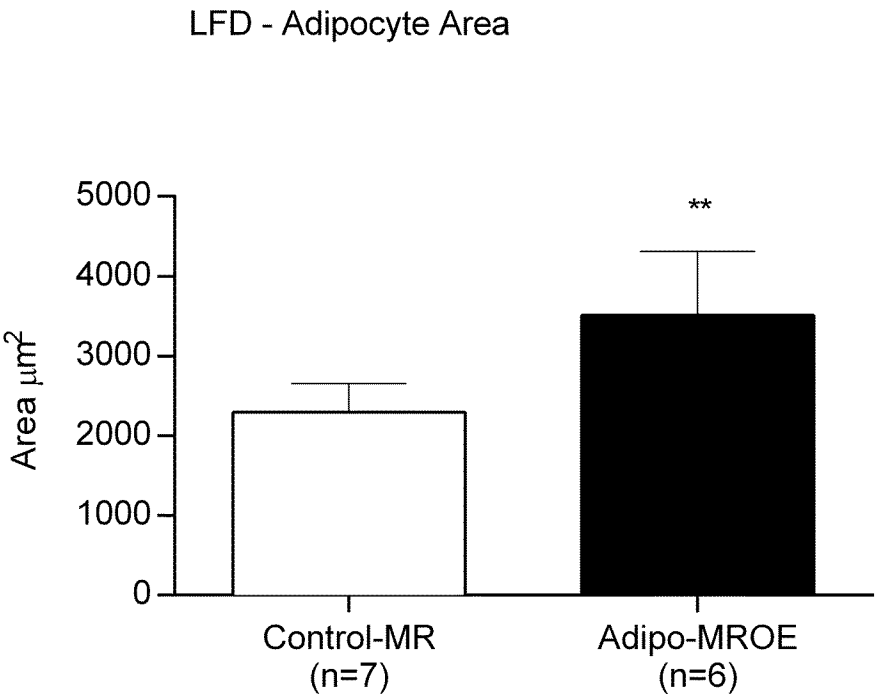


Figure 3A



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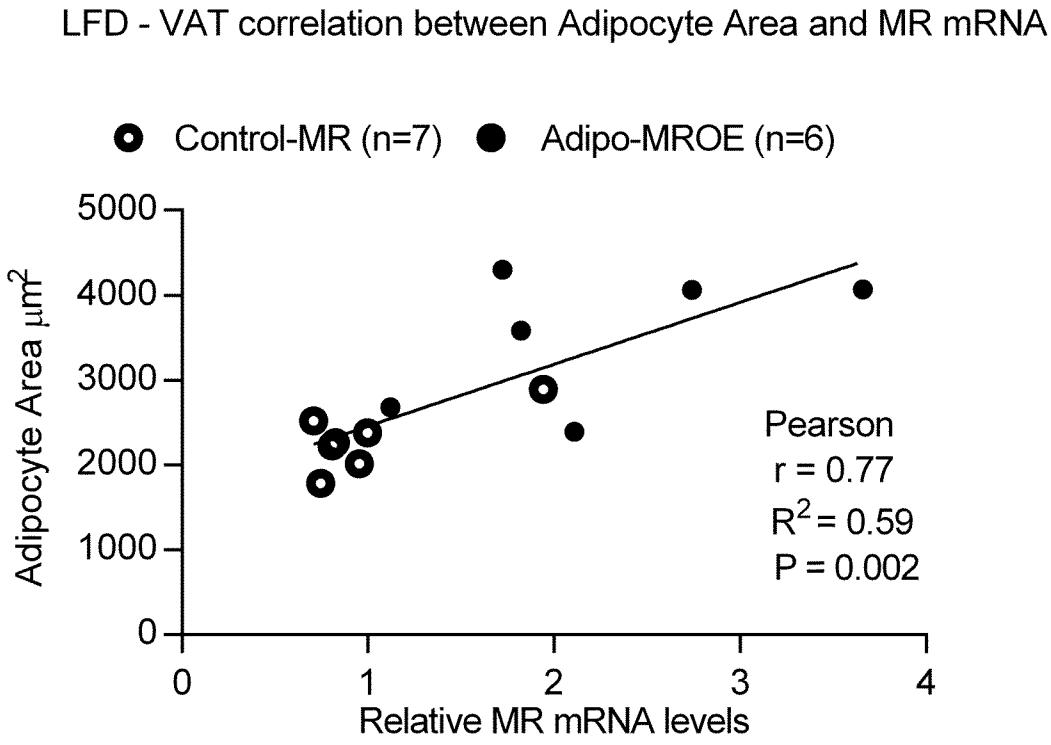

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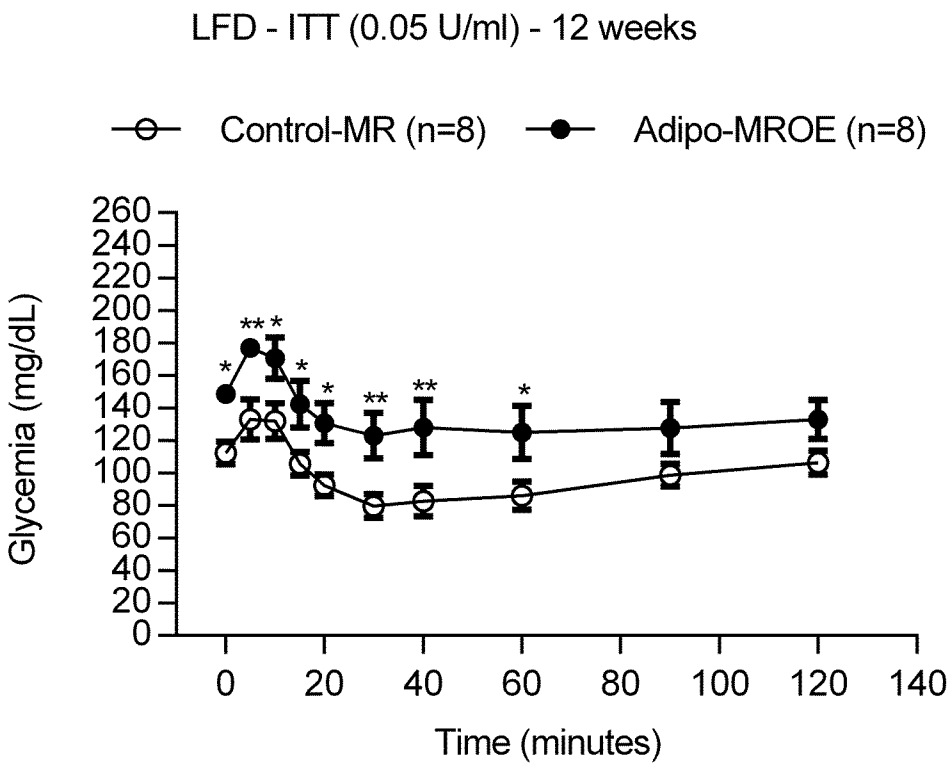
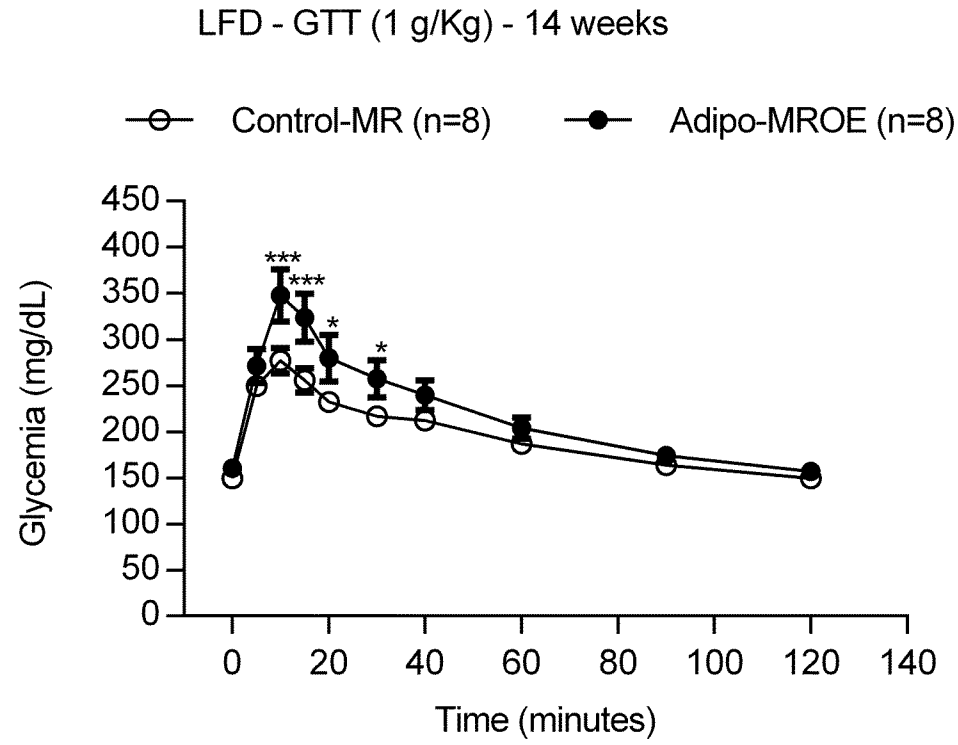


Figure 3D



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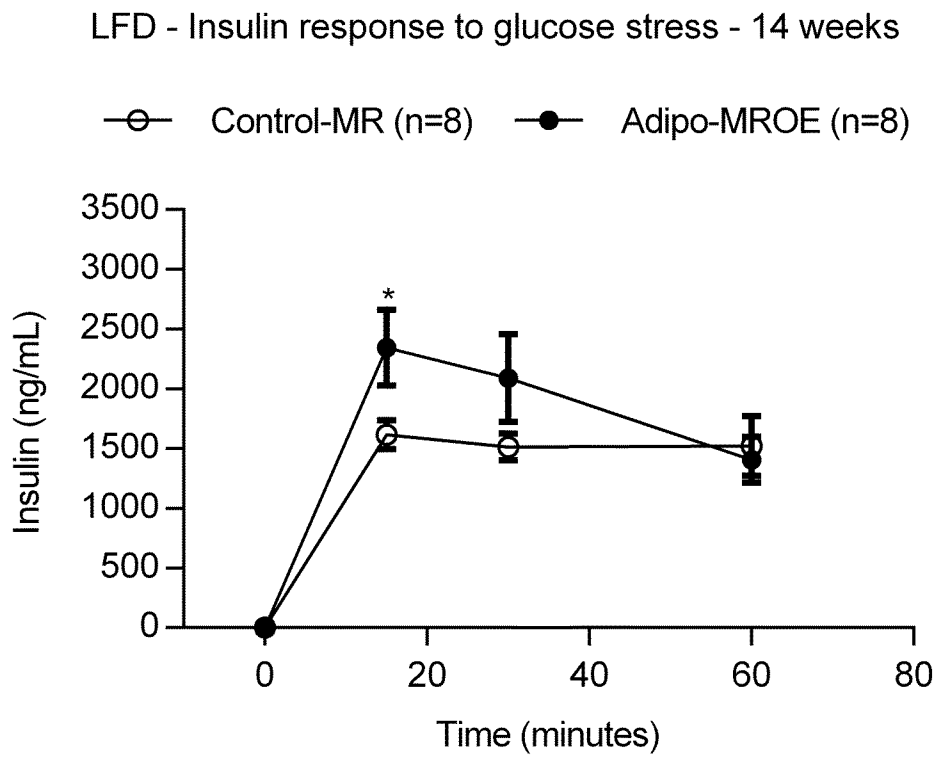

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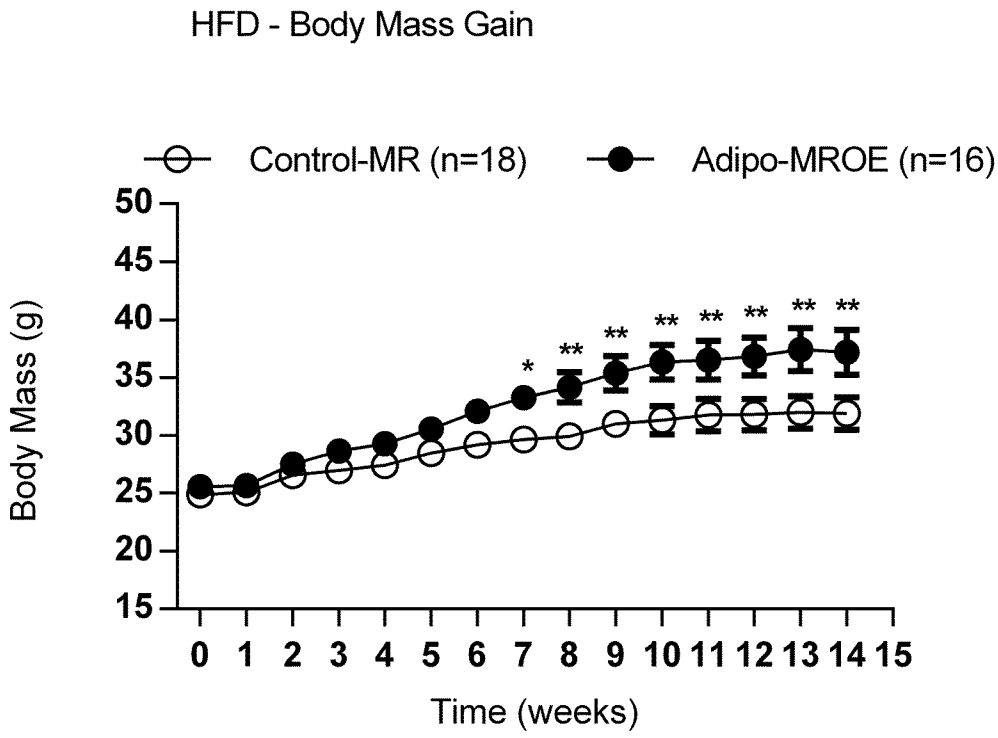
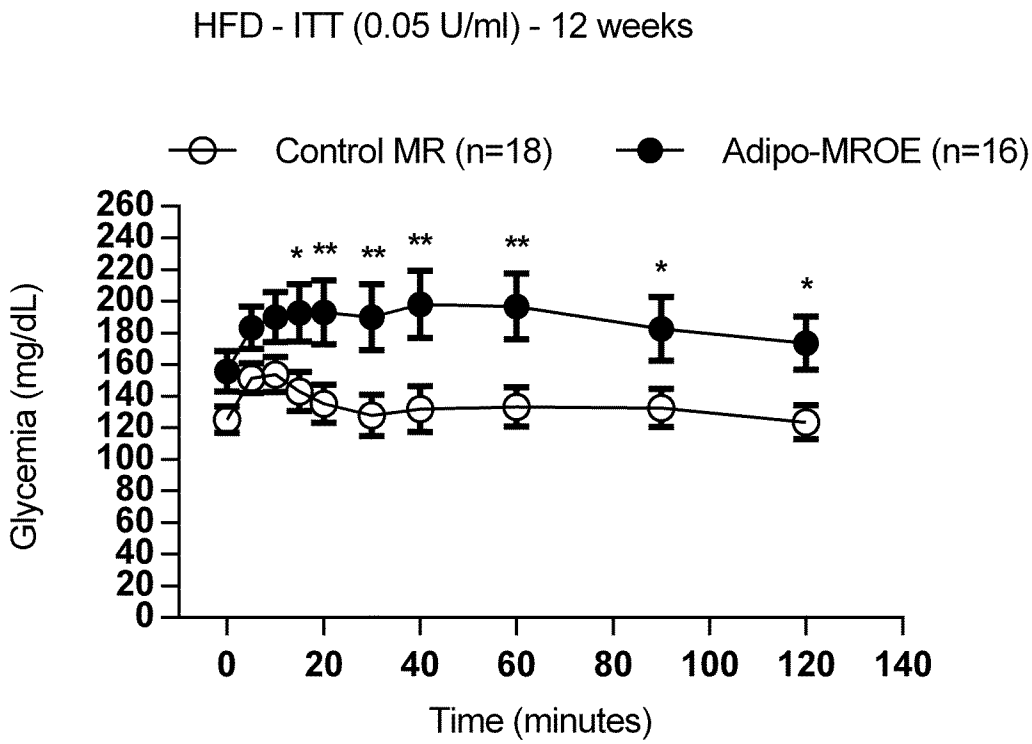


Figure 4A



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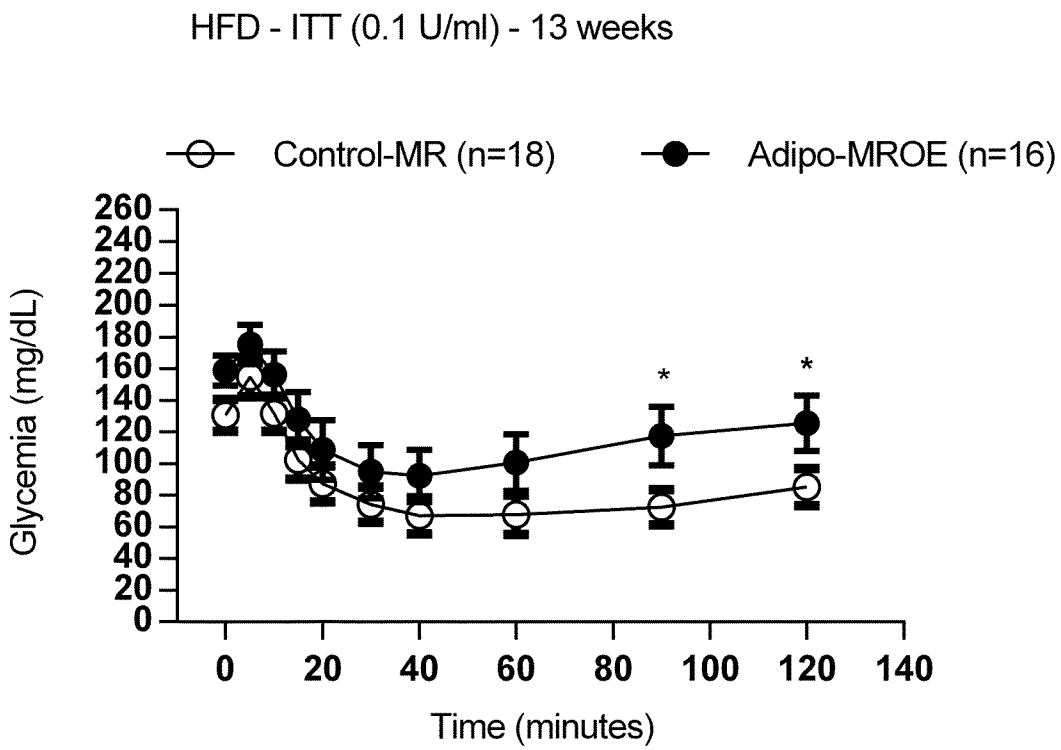


Figure 4C

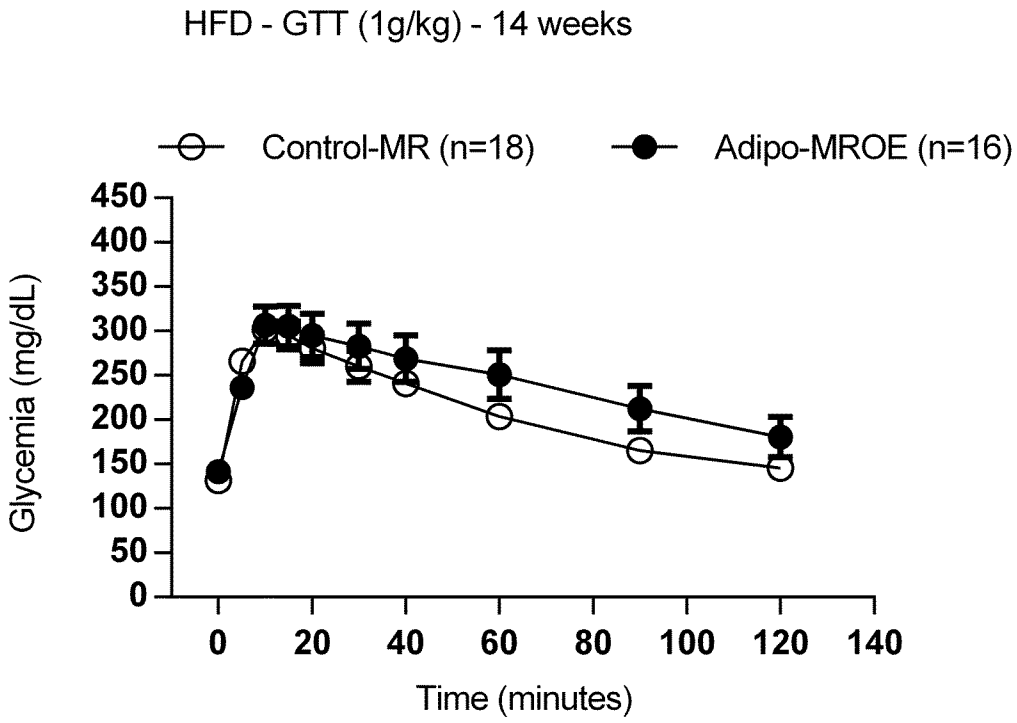


Figure 4D

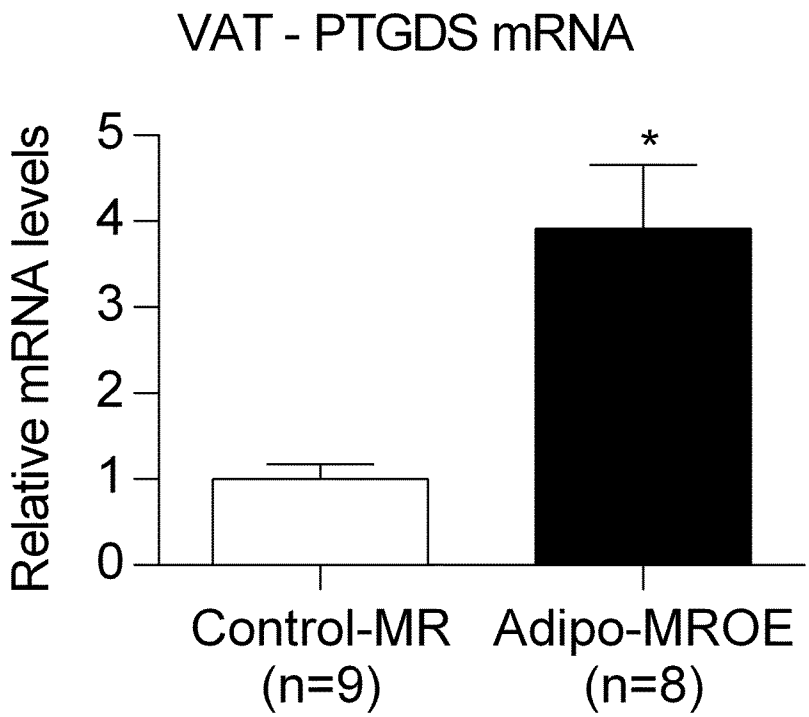


Figure 5A

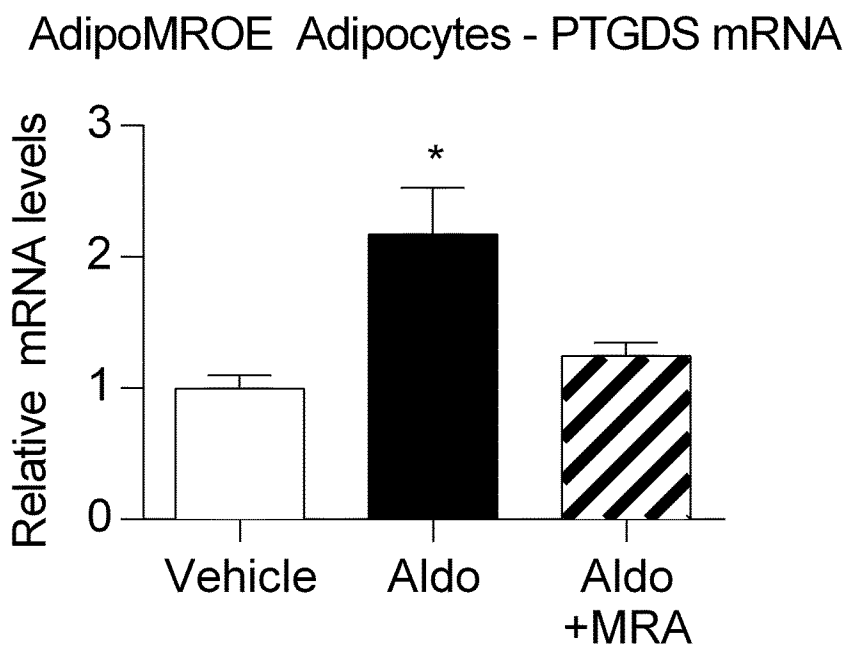


Figure 5B

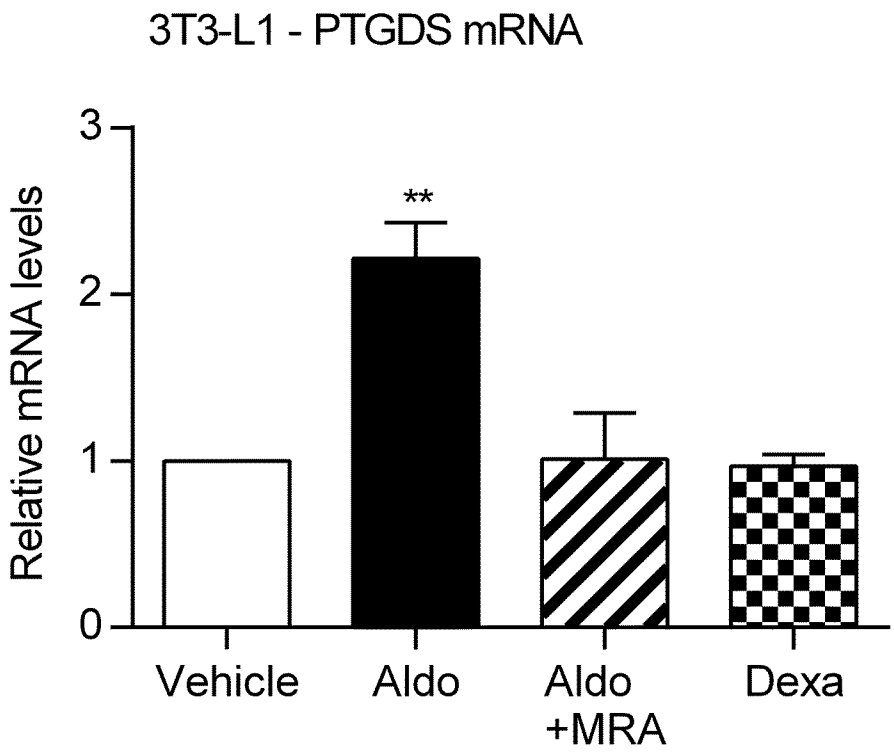


Figure 5C

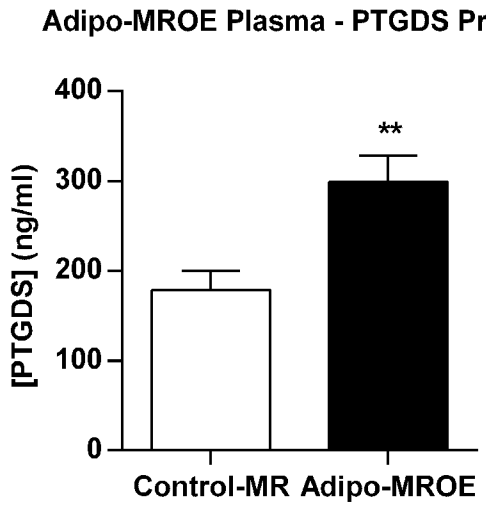


Figure 5D

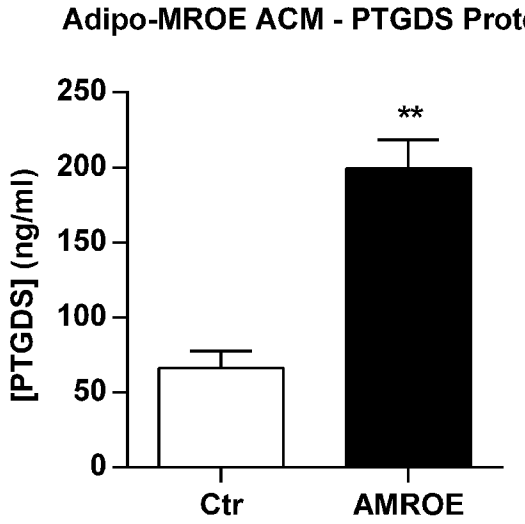


Figure 5E

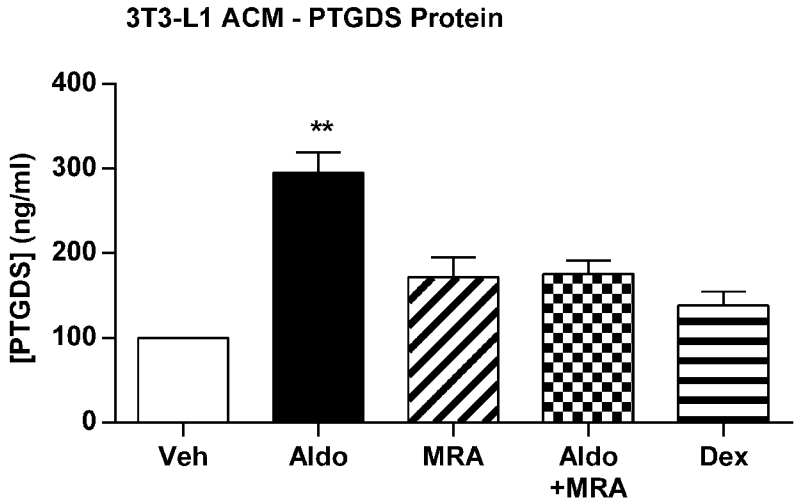
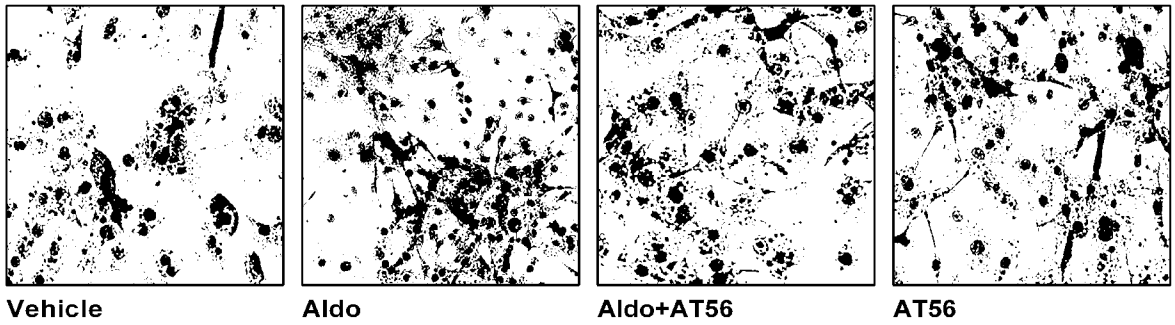


Figure 5F



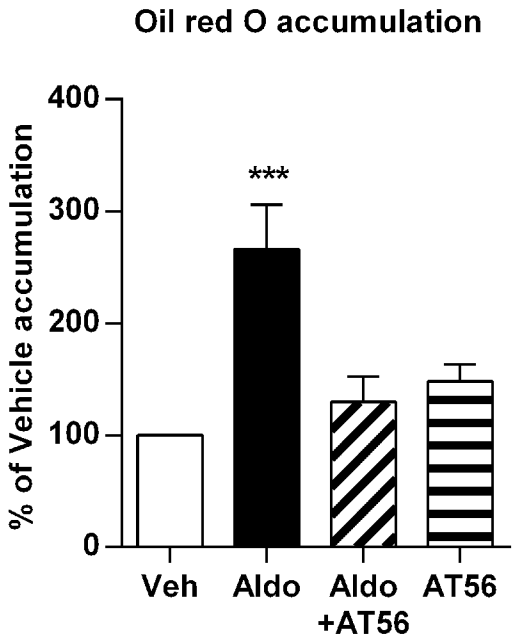


Figure 6A

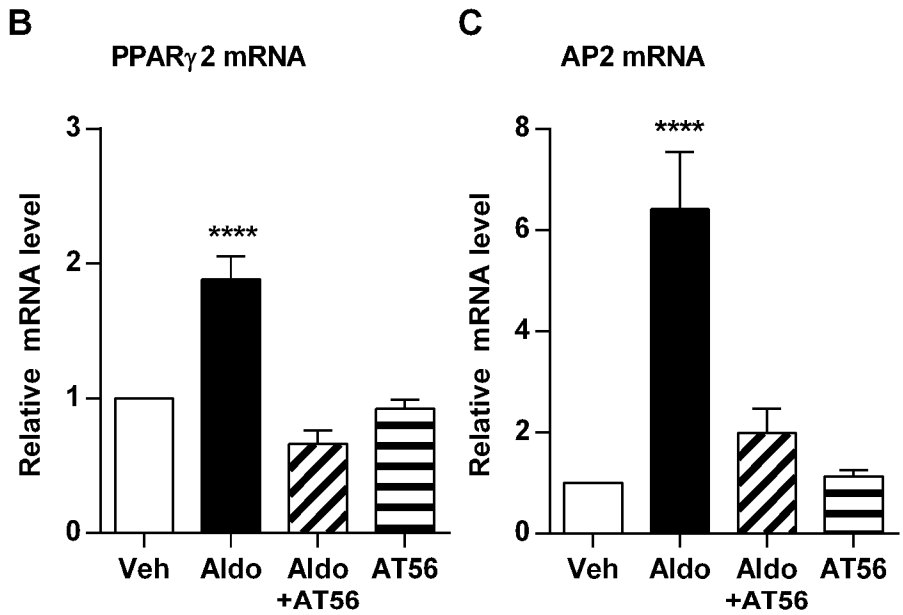


Figure 6B-C

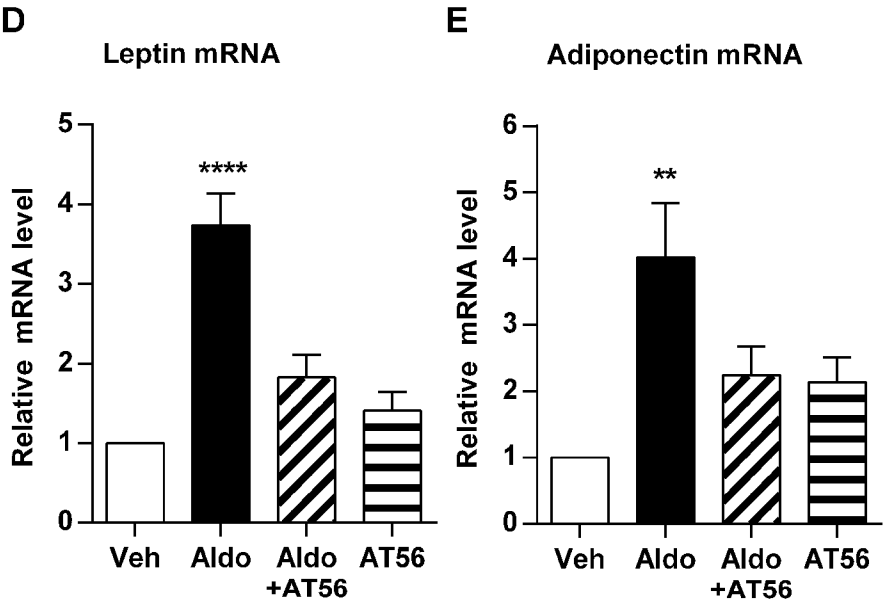
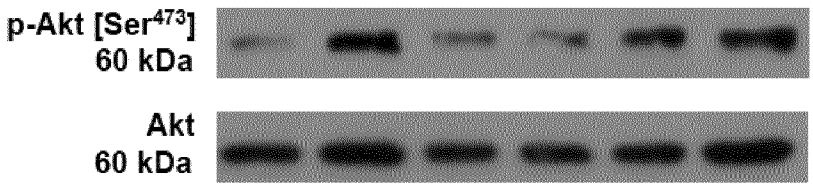


Figure 6D-E



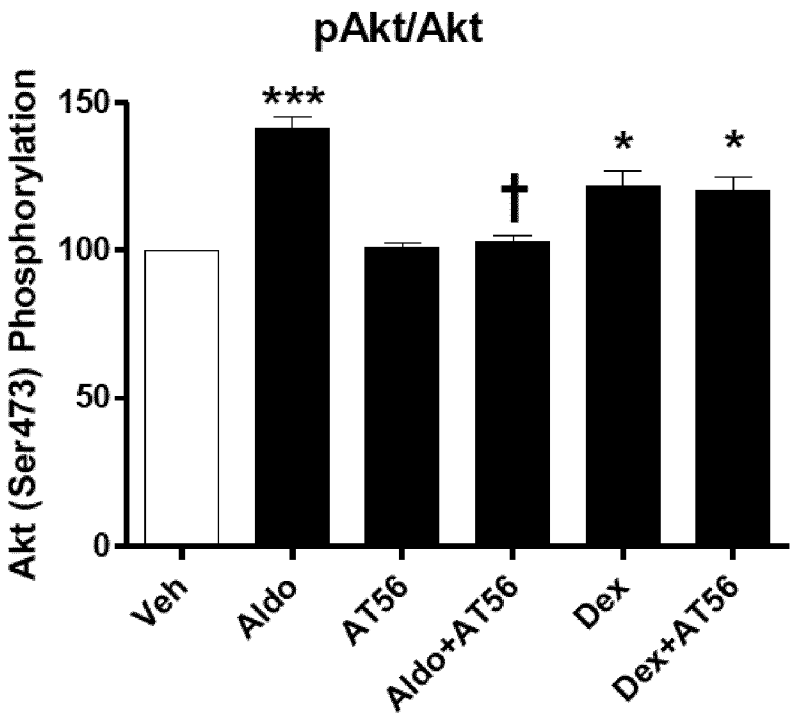


Figure 6F

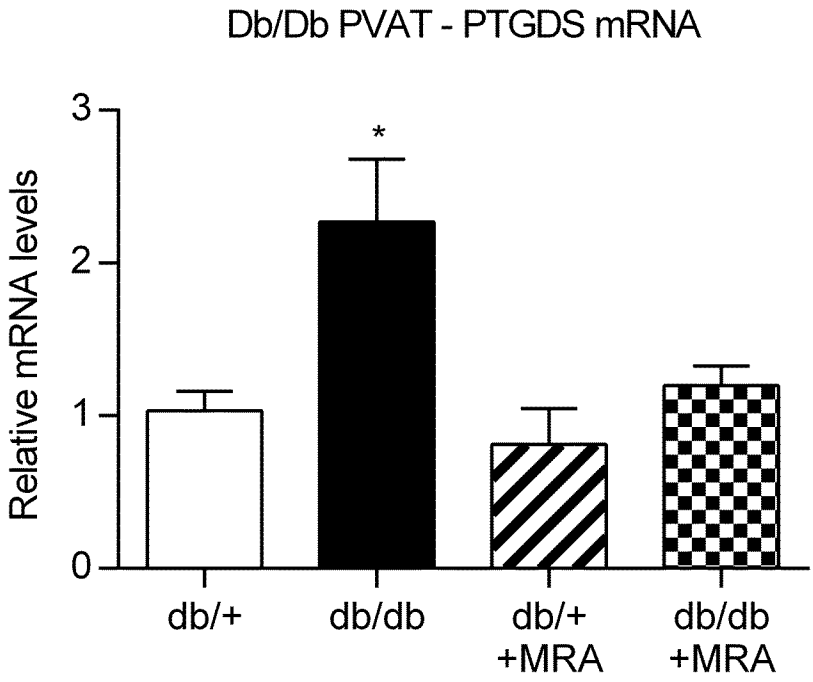


Figure 7A

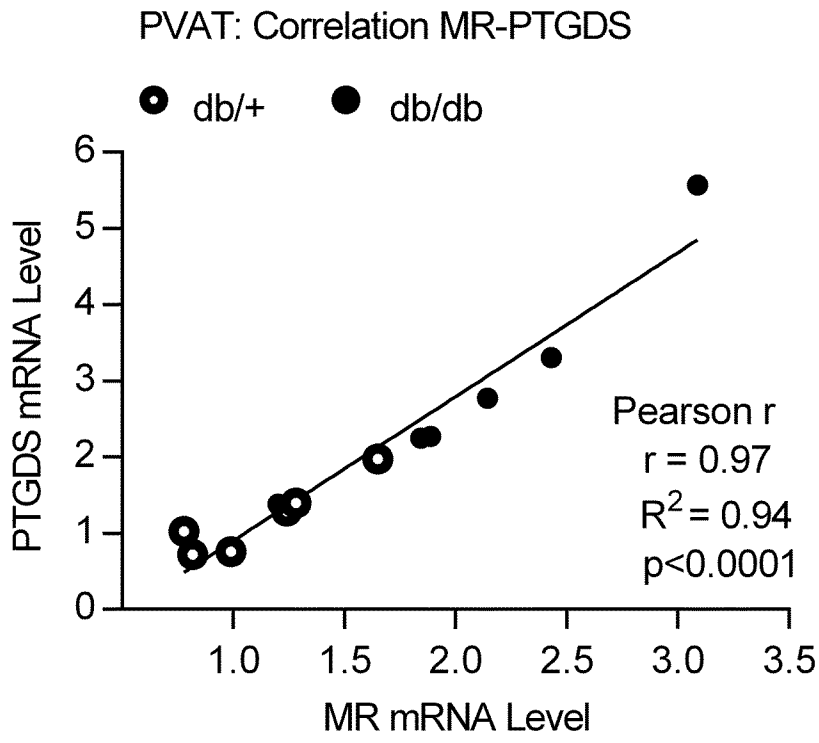


Figure 7B

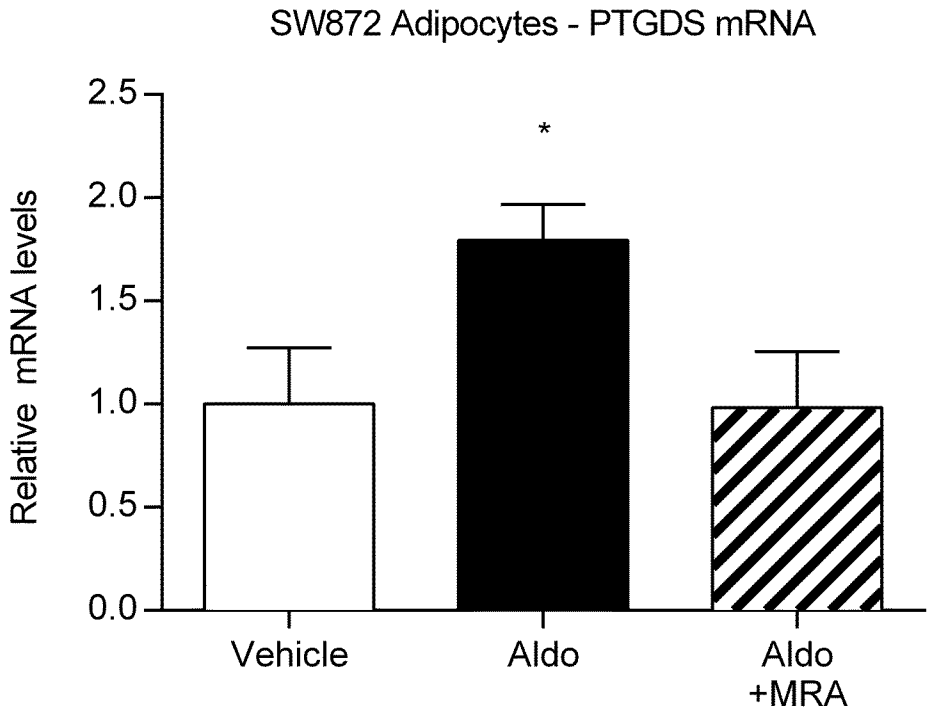


Figure 8A

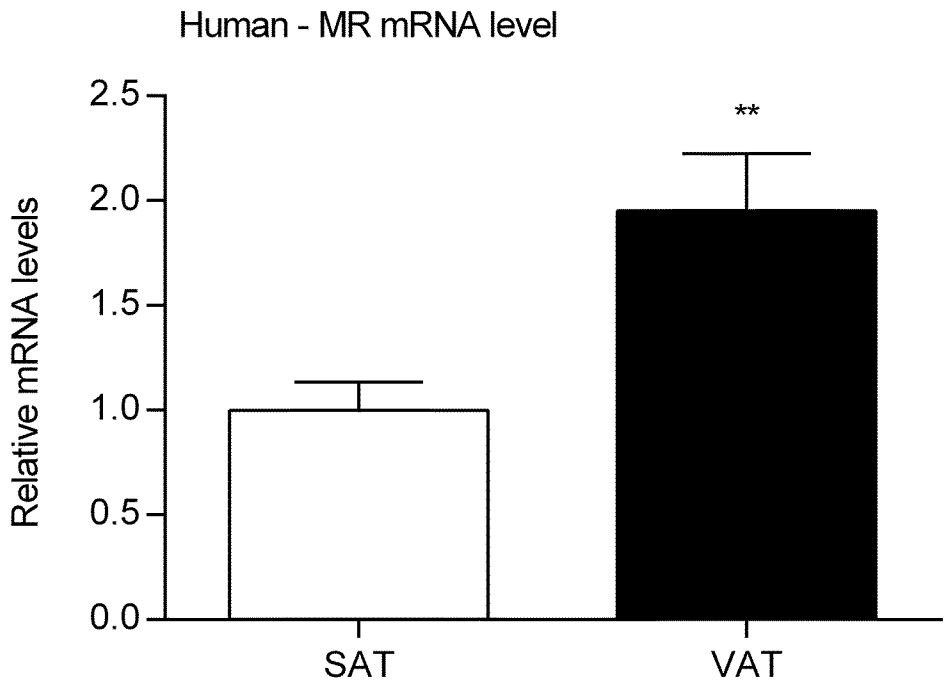


Figure 8B

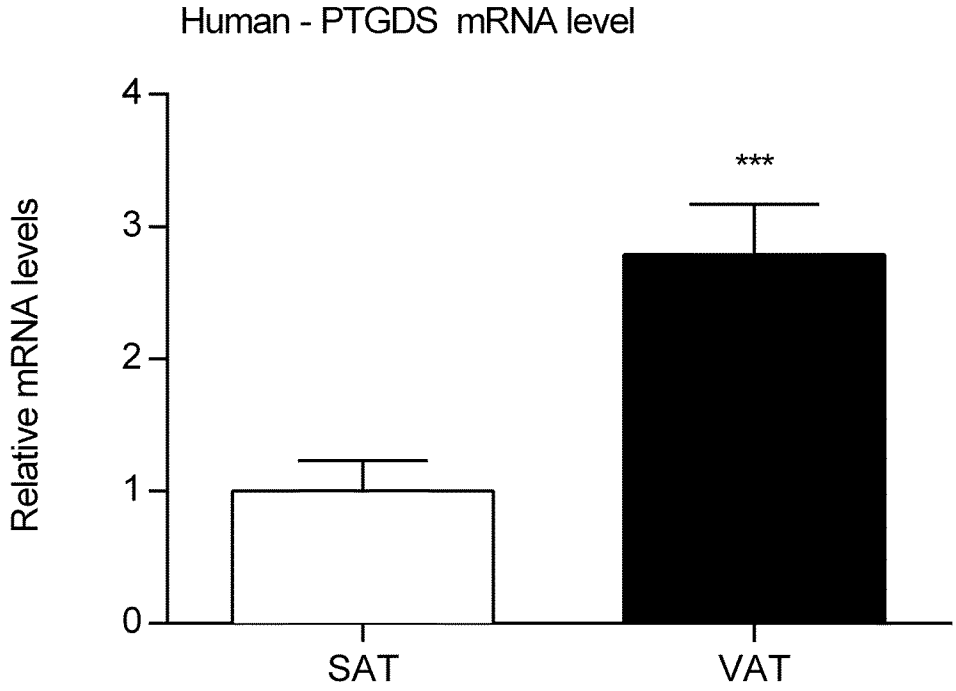


Figure 8C

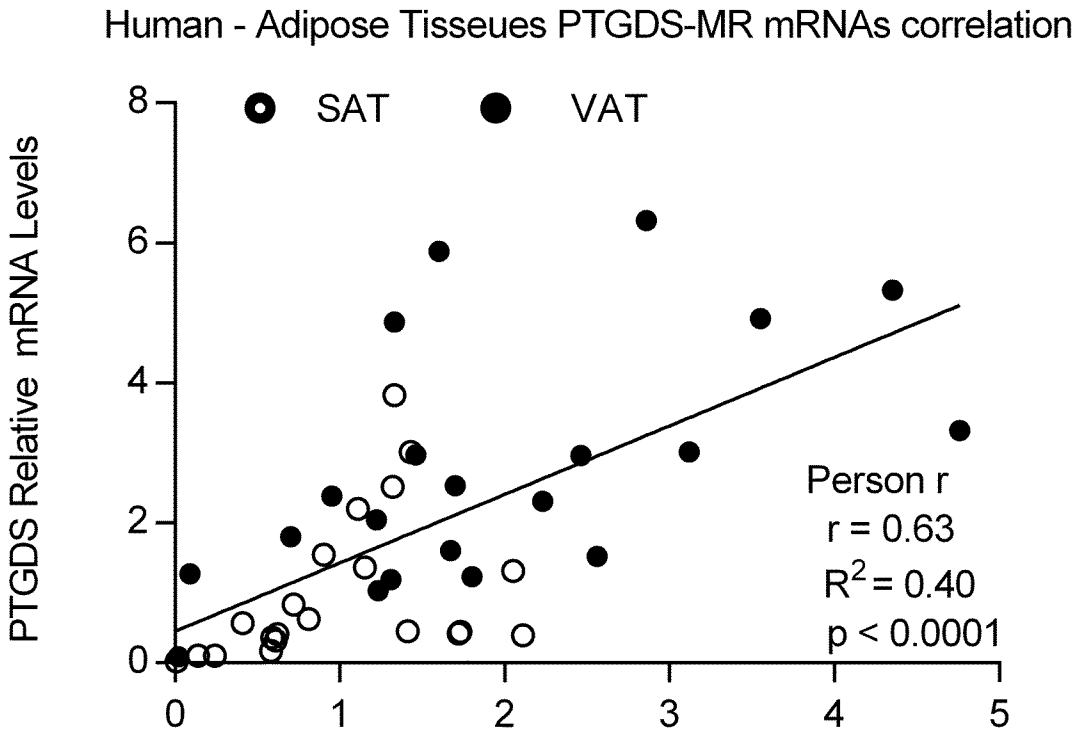


Figure 8D

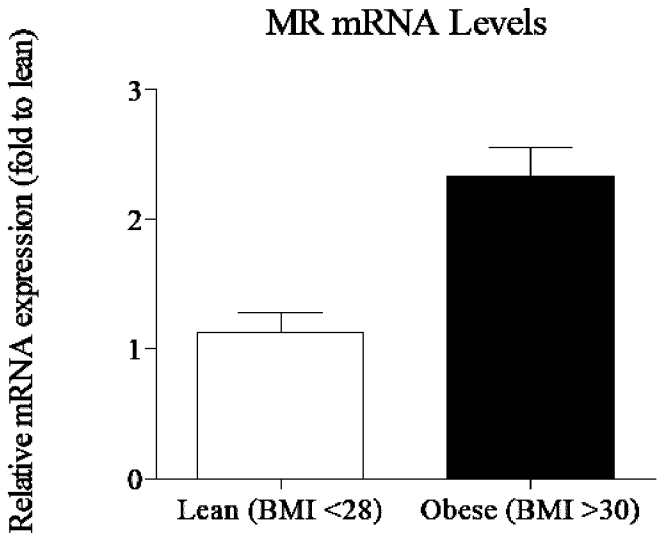


Figure 9A

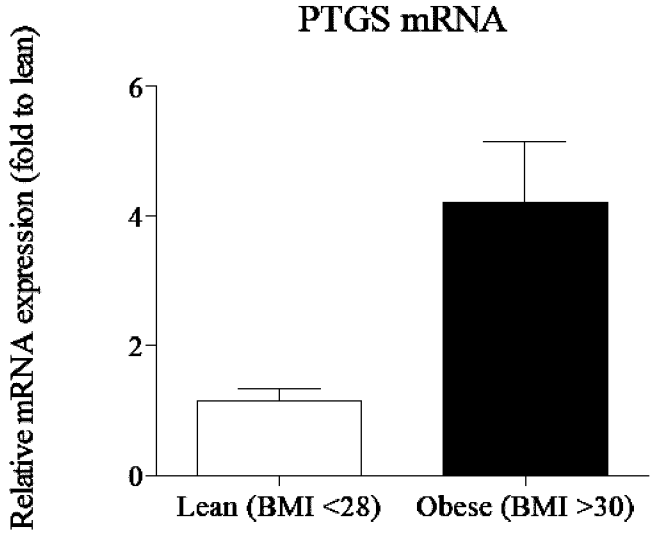


Figure 9B

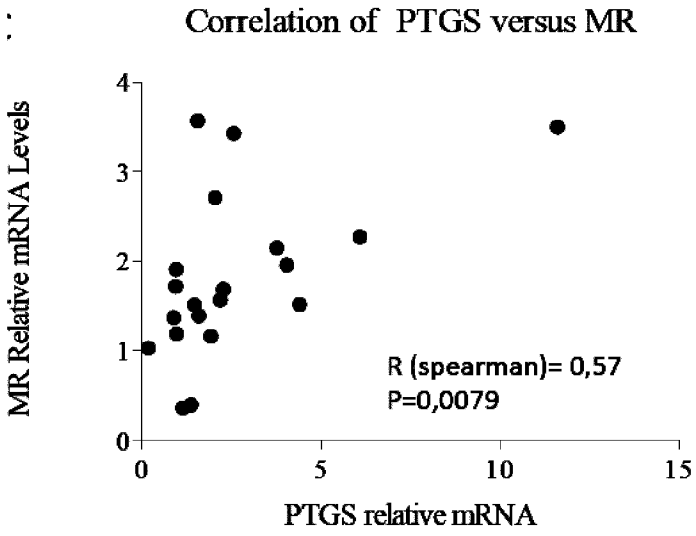


Figure 9C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/065550

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/52 A61K31/585
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)
C12Q G01N A61K

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HIRAWA N ET AL: "URINARY PROSTAGLANDIN D SYNTHASE (BETA-TRACE) EXCRETION INCREASES IN THE EARLY STAGE OF DIABETES MELLITUS", NEPHRON, S. KARGER AG, SWITZERLAND, vol. 87, no. 4, 1 April 2001 (2001-04-01), pages 321-327, XP009065057, ISSN: 0028-2766, DOI: 10.1159/000045937 abstract ----- A. HIRATA ET AL: "Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice", CARDIOVASCULAR RESEARCH, vol. 84, no. 1, 8 June 2009 (2009-06-08), pages 164-172, XP055157266, ISSN: 0008-6363, DOI: 10.1093/cvr/cvp191 abstract ----- -/--	1-10 1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 September 2015	30/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Knudsen, Henrik

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Form PCT/ISA/210 (second sheet) (April 2005)

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A	QUINKLER M ET AL: "Depot-specific prostaglandin synthesis in human adipose tissue: A novel possible mechanism of adipogenesis", GENE, ELSEVIER, AMSTERDAM, NL, vol. 380, no. 2, 10 June 2006 (2006-06-10) , pages 137-143, XP024934612, ISSN: 0378-1119, DOI: 10.1016/J.GENE.2006.05.026 [retrieved on 2006-10-01] page 141, right-hand column, paragraph 1	6
A	EP 1 224 861 A1 (JAPAN SCIENCE & TECH CORP [JP]; OSAKA BAIOSCIENCE INST [JP]; ORIENTAL) 24 July 2002 (2002-07-24) claim 5; example 4	1-14
A	ABU ASAD CHOWDHURY ET AL: "Sustained expression of lipocalin-type prostaglandin D synthase in the antisense direction positively regulates adipogenesis in cloned cultured preadipocytes", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 411, no. 2, 24 June 2011 (2011-06-24) , pages 287-292, XP028296870, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2011.06.126 [retrieved on 2011-06-24] abstract	1-14

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KO FUJIMORI ET AL: "Activation of adipogenesis by lipocalin-type prostaglandin D synthase-generated -PGJacting through PPAR -dependent and independent pathways", GENE, ELSEVIER, AMSTERDAM, NL, vol. 505, no. 1, 28 May 2012 (2012-05-28), pages 46-52, XP028426559, ISSN: 0378-1119, DOI: 10.1016/J.GENE.2012.05.052 [retrieved on 2012-06-01] abstract ----- ANDO K ET AL: "Metabolic syndrome and oxidative stress", FREE RADICAL BIOLOGY AND MEDICINE,, vol. 47, no. 3, 3 May 2009 (2009-05-03), pages 213-218, XP026196320, ISSN: 0891-5849, DOI: 10.1016/J.FREERADBIOMED.2009.04.030 [retrieved on 2009-05-03] page 215 ----- S. VIRTUE ET AL: "A New Role for Lipocalin Prostaglandin D Synthase in the Regulation of Brown Adipose Tissue Substrate Utilization", DIABETES, vol. 61, no. 12, 24 August 2012 (2012-08-24), pages 3139-3147, XP055157518, ISSN: 0012-1797, DOI: 10.2337/db12-0015 page 3140, left-hand column, paragraph 1 ----- WO 2010/046411 A1 (INST NAT SANTE RECH MED [FR]; JAISSER FREDERIC [FR]; FARMAN NICOLETTE) 29 April 2010 (2010-04-29) claims 1,12 ----- URBANET RICCARDO ET AL: "Adipocyte Mineralocorticoid Receptor Activation Leads to Metabolic Syndrome and Induction of Prostaglandin D2 Synthase.", 11 May 2015 (2015-05-11), HYPERTENSION JUL 2015, VOL. 66, NR. 1, PAGE(S) 149 - 157, XP9186022, ISSN: 1524-4563 DOI: 10.1161/HYPERTENSIONAHA.114.04981, the whole document -/--	1-14 1-14 1-14 1-14 1-14

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y,P	S VIRTUE ET AL: "Prostaglandin profiling reveals a role for haematopoietic prostaglandin D synthase in adipose tissue macrophage polarisation in mice and humans", INTERNATIONAL JOURNAL OF OBESITY, vol. 39, no. 7, 24 March 2015 (2015-03-24), pages 1151-1160, XP055212397, ISSN: 0307-0565, DOI: 10.1038/ijo.2015.34 abstract -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/065550

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