

PROJECT FINAL REPORT

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1. Publishable summary

1.1 Executive summary.

Obesity is one of the most serious and fast-growing health problems in the European Union, and a leading cause of diabetes. The main barrier for approval of an anti-obesity drug is the safety requirements that led to marketing prohibition of almost all anti-obesity drugs approved in the latest period of time such as Sibutramine or Rimonabant. This situation has led to a status on which one of the most relevant causes of morbid-mortality in humans is almost devoid of effective pharmacotherapeutic alternatives. There is an urgent need for new drugs against obesity that is reflected in the EU VII FP research call for projects under the topic HEALTH-2007-2.4.3-6: *Nutritional signals and the development of new diabetes/obesity therapeutic agents. This project will focus on the effects of alternative compounds which improve carbohydrate/lipid metabolism or modify body weight, and could be used in the development of new therapeutics in the treatment of hyperglycaemia and hyperlipidemia.* To be successful, any research proposal has to discover novel or improved treatments in the shortest possible timeframe. The FP VII project REPROBESITY (www.reprobesity.eu) proposed to overcome the barriers around obesity by discovering

- a) New indications of existing drugs with proven safety profiles as anti-obesity therapies. Since a relevant clinical end-point for an anti-obesity drug is its ability to reduce abdominal fat, the project has focused on approaches targeting directly abdominal fat cells. This is accomplished by a new specific technology that allows ex-vivo monitoring by flow cytometry techniques of adipose cells responses to libraries of approved drugs. This technology developed by VIVIA Biotech, is followed by target selection and pharmacological validation performed by a team of European Community researchers specialized on top quality preclinical studies in obesity.
- b) Phenotypes and biomarkers that identify subsets of patients with safe and efficacious responses to drugs. The biomarker project intend to establish if we can obtain a way of identify the responding patients to a given therapy against obesity, since the experience with formerly approved drugs indicates that its utility is limited to a restricted set of patients. A new phenotype and/or biomarker may identify responsive patients with good safety profiles.

The work performed by the *Reprobesity* consortium has allowed to:

- a) Develop for the first time an effective technique for the reprofiling of existing drugs using both, ex vivo samples of human adipose tissue and human cells engineered to express selected pharmacological targets to identify both, new drug indications and new chemical entities. This approach has allowed the filing of 9 industrial patents derived of project's outcomes.
- b) Develop a new technique of combinatorial cytomic biomarkers capable of identify the contribution of pathological conditions (i.e. obesity) and environmental factors (i.e. consumption of carbohydrates), to an abnormal cellular response (i.e. reactive oxygen species production, expression of the insulin receptor, expression of the glucose transporter or expression of the fatty acid transporter) that may account for the pathological consequences of obesity (i.e. metabolic syndrome, diabetes etc..).
- c) Identify the pathophysiological role of biochemical signalling pathways on obesity. This information is useful to develop new therapeutic alternatives. These targets include known receptors such as GLP-1 receptor, peripheral cannabinoid CB1 receptors, β 3-adrenergic receptors, Thyroid hormone, as well as several orphan receptors, including GPR55, nuclear receptors such as PPAR α receptors, and signalling pathways such as that of Sirtuin1/p53.
- d) Patent and development of new chemical entities interacting with these targets, one of them, an allosteric modulator of GLP-1 receptors, being ready for clinical trials in humans.

1.2 Summary description of the project context and the main objectives.

1.2.1 Context: The epidemics of obesity

Obesity incidence in European countries is a first magnitude problem for health systems as the enormous implications for health; mortality and quality of life have become apparent. Obesity is the major risk factor for cardiovascular disorders and type 2 diabetes and is also independently associated with increased mortality and morbidity. Obese individuals die prematurely from coronary and cerebrovascular disease as well as certain cancers. In addition, they suffer substantial morbidity and reduced quality of life from musculoskeletal and gastrointestinal disease, depression and social isolation and poor mobility. We are now starting to understand how obesity emerges as an allostatic disorder on which reference set points of the organism marking ideal body weight and energy expenditure, start a random allostatic shift towards unstable ever growing body weight and reduced energy utilization. These allostatic shifts are probably related with the induction of profound alterations in the phenotype of the cells involved in metabolic control, specially the adipose tissue, the muscle, the hepatocytes and the central neurons of the hypothalamic area. These phenotypic changes are due to genetic and epigenetic modifications yet to be clarified. Such physiological mechanisms begin to provide an explanation for the poorly sustained efficacy of purely behavioural approaches to the problem of obesity, particularly when it is severe. To cure obesity, (and thereby control the incidence of diabetes and reduce cardiovascular morbidity) we must find a way to pharmacologically modulate this allostatic shift, restoring the normal phenotype and keeping it stable against the external pressures. The question is where in the body and with which tools, i.e. new drugs.

Current pharmacological treatments are no more consistently effective than dieting. In fact, pharmaceutical industry has failed to find new drugs capable of reducing body weight in a sustained way. Moreover, candidates targeting central hypothalamic set point-related circuits have failed to provide molecules with proven efficacy against obesity. Only the cannabinoid CB1 receptor blocker Rimonabant has been approved in the latest years as a drug against obesity, and the reason lies in their multifactorial mechanism of actions, since its target, the CB1 receptor, is present in almost all the stages of the energy expenditure/appetite controlling circuits. While failing therapies have classically been designed to target central neuronal systems like the noradrenergic and serotonergic systems of the brain, diffuse systems that influence a wide range of bodily functions, peripheral targets may offer new alternatives. The success of the CB1 receptor blocker illustrates the need of targeting peripheral tissues, specially the adipose tissue, since the complexity of the homeostatic circuits in the hypothalamus guarantees a defensive neuroadaptive response that may abolish the efficacy of drugs targeting central nervous system mechanisms.

The adipocyte as a pivotal cell in energy homeostasis and body weight

As stated above, we can identify the adipocyte as a major target for the development of anti-obesity therapies. Rather than simply passively storing excess energy as triglycerides, the adipocyte has emerged as a highly active participant in the processes of energy balance, nutrient partitioning and insulin sensitivity. The adipocyte secretes peptide and nonpeptide hormonal products which are active participants in the control of energy balance (e.g. leptin, IL-6) and insulin sensitivity (e.g. glucocorticoids, adiponectin, TNF α , resistin). Excessive secretion (or in the case of adiponectin, defective production) of one or more of these products from the adipocytes of obese subjects may lead directly to the insulin resistance which precedes type 2 diabetes. Through the activity of brown adipocytes, fat tissue can actively promote non-shivering thermogenesis through uncoupling of oxidative phosphorylation. Finally, the process of new fat cell acquisition can be actively manipulated throughout life by signals converging on key nuclear receptors. PPAR γ (peroxisome proliferator-activated receptor γ) is a nuclear receptor with a major influence on both adipogenesis and insulin sensitivity and has become of great clinical significance as it is the target for the thiazolidinedione group of antidiabetic drugs. Adipocytes are capable of generating their own steroid ligands through hormonal interconversion and over expression of steroid interconverting enzyme in fat can recapitulate most of the features of central obesity and type 2 diabetes.

1.2.2 Objectives

The present project is aimed to give answer to the needs stated in the work program and anticipated in the above introduction: the search of new effective therapeutics against obesity, including the implementation of highly selective procedures for phenotyping subsets of patients. This will be accomplished by means of the use a modern ex-vivo cytomic platform, ExviTech, developed by the SME VIVIA Biotech. The platform will be used to:

- 1) Identify biomarkers of complicated obesity in whole blood samples and abdominal adipocytes from animal models of obesity and obese patients, using a novel approach called Combinatorial Cytomic Biomarkers.
- 2) Searching for new indications of drugs (given alone or in combination) already tested in humans in order to identify those who can modify abdominal adipocyte physiology, by lowering their fat content via lypolysis or thermogenesis, or by lowering their glucose uptake.

A consortium of basic and clinical investigators of 4 European countries is organized to provide to the company:

- a) research models and animal samples on which validate both drug candidates and blood biomarkers,
- b) patient samples for ex-vivo testing and
- c) patient phenotyping for a better definition of new drug indications.

The final goal of the project is to generate two types of biomedical products, cytomic biomarkers of complicated obesity measured in ex-vivo samples, and candidates for new indications of existing drugs suitable for progressing towards clinical trials for the treatment and prevention of complicated obesity. The research, technological development and innovation activities have been broken down into two blocks of work:

Workblock A: Reprofilng drugs targeting the abdominal adipose tissue (Workpackages 1 to 3)

Workblock B: Combinatorial Cytomic Biomarkers of obesity in fresh whole blood and abdominal adipocytes (Workpackages 4 to 6)

OBJECTIVES:

Objective 1 Identify new indications of existing drugs, alone or in combination, with potential anti-obesity efficacy by lowering the fat content and the glucose uptake of abdominal fat cells, which would be expected to improve carbohydrate/lipid metabolism and lower body weight, extracted from the above phenotyped patients. This will be accomplished by screening ex vivo approximately 2.000 known drugs against adipocytes using the novel technology platform “ExviTech” from VIVIA Biotech.

Objective 2 Candidates derived from the objective 1 would be evaluated in animal models of obesity. Loss or blockade of body weight gain will be studied in these animal models, and in transgenic mice bearing mutations in genes leading to obese or lean phenotypes.

Objective 3 Compounds fulfilling objectives of WP1 and WP2 will be evaluated in order to establish their mechanism of action. Molecular biology, biochemistry, cellular physiology, systems physiology with special emphasis on endocrinology, a molecular and systems pharmacology will be used as systematic approaches to elucidate the mechanisms of action of the effective drugs.

Objective 4. To develop the concept of *ex vivo* Combinatorial Cytomic Biomarkers (CCB) in freshly isolated blood and adipocytes samples of animal models of obesity. Selecting candidates for biomarking obese phenotypes

Objective 5. To discover biomarkers for subsets of obese patients that may correlate with therapeutical outcomes. These biomarkers will be discovered by a novel approach called Combinatorial Cytomic Biomarkers developed by VIVIA Biotech, applied to cells from two physiologically interlinked sources: blood and abdominal-fat samples,

extracted from the above phenotyped patients. Additional approaches for biochemical biomarkers shall be taken in consideration.

Objective 6. To establish the functional significance of biomarkers. Use of animal models to link the efficacy of top candidates tested with the modification of Combinatorial Cytomic Biomarkers. To perform a comparison with a drug of proved efficacy (Cannabinoid Receptor antagonist).

Objective 7. To achieve clinical phenotyping of obese patients. The aim is to identify those that would benefit from existing therapies such as Rimonabant. Additional objectives include the generation of a data base with biomarkers useful for phenotyping, adipocyte response to reprofiling drugs and clinical data of selected patients. The final goal is the proposal of new candidates for clinical trials in obesity.

1.1 Progress beyond the state-of-the-art: Reprofilng as a rapid and efficient strategy for identification of new therapeutic indications

The reprofiling model to identify new therapeutic agents is a modern biotechnology approach which is shorter and safer. Research with this system uses previously approved therapeutic agents for human use (phase II and up), shortcutting the riskiest phases (pre-clinical phases and phase I) which represent the principal cause of failure with only 1 in 5.000 agents passing this phases, shortening the time the therapeutic agent arrives the market from 15 to 5 years. The result is a therapeutic agent candidate for clinical phase II, an inflexion point in the current pharmaceutical market for its lower risk since only 1 in 5 agents are finally approved for marketing. Even though we present this as a novel model, 50% of currently approved agents come from reprofiling, discovered one at a time, such as the cardiovascular indication for aspirin, in patient samples, process that VIVIA Biotech pretends to escalate to thousands of therapeutic agents at once.

Reprofiling strategies might be specially suited to discover anti-obesity drugs because of their known and validated safety profile. The huge numbers of the potential patient population makes safety a primary criterion to approve an anti-obesity drug. The reason is that small percentages of patients with severe adverse effects could represent large numbers of patients in the community. This was the reason for the removal of the arthritis drug Vioxx, which has led to enhanced safety standards for approval of drugs for large indications. The advantage of reprofiling strategies over the discovery of new chemical entities is the known and often validated safety profile of existing drugs that have been already tested in at least Phase II clinical trials.

The differential factors that give us an upper hand in competing with other enterprises and research groups that work in new indications for obesity with existing drugs (reprofiling) are the following:

1. **Technologic Novelty:** The Technological Platform ExviTech (ex-vivo Technology), which allows in a fresh biological patient sample the exvivo analysis of the effect of thousands of therapeutic agents at a time in a routine and systematic way. In comparison, existing technologies can only analyse the effect of 100 to 200 agents at a time. The first innovative component of our technology is the automatization of its central instrument, flow cytometry. Its second component is its integration in an industrial chain that allows to operate on an industrial scale.
2. **Efficacy. Researching thousands of components in fresh patient samples:** The majority of enterprises develop therapeutic agents based on genes and therapeutic targets specifically expressed in laboratory cells, not in ex vivo samples. Some are testing a few agents on ex vivo samples but none is applying screening of thousands of agents by sample like Vivia Biotech. This capacity of evaluation thousands of agents at a time on a single sample will allow us to identify better opportunities for treatment. A key biomarker of complicated obesity is the waist size, directly correlated with cardiovascular risk and related to other diseases such as diabetes. Consequently, new anti-obesity drugs have to diminish the abdominal fat content. Because this fat is accumulated in fat cells called adipocytes, we propose to screen known drugs directly on freshly extracted adipocytes from abdominal fat tissue.

3. **Opportunity. The ExviTech platform is uniquely positioned to screen for the best possible mechanism of losing weight.** Burning abdominal fat by Metaplasia. Converting fat (lipids) into heat is a cleaner and safer process to lose weight than the chemical breakdown of fat (lipids) that yields potentially dangerous byproducts in significant concentrations. The type of adipose tissue called brown fat is made of cells filled with a very special variety of mitochondria and small droplets of fat; these mitochondria are able to consume energy to produce heat. They can do this thanks to a specific mitochondrial uncoupling protein (Ricquier et al. *Reprod. Nutr. Dev.* 1985;25: 175-181; Ricquier et al. *J. Am J Physiol* 1983; 245: C172-177), which uncouples oxidative phosphorylation and allows the respiring brown fat to become a “heat gland”. As may be expected in rats, this protein is produced in greater amounts by exposure to cold (Bouillaud et al. *J Biol Chem* 1984; 259:11583-11586). Now, brown fat is richly supplied with sympathetic nerves. If these nerves are severed, the brown fat turns into white fat; conversely, in patients bearing a pheochromocytoma (a tumor that secretes epinephrine and other catecholamines), the perirenal fat may turn from white to brown (Lean et al. *Int J Obesity* 1986;10:219-227; Melicow MM, *Arch Pathol*; 1957: 63: 367-372). The same effect has been observed in rats (Ricquier et al. *Reprod. Nutr. Dev.* 1985;25: 175-181; Ricquier et al. *J. Am J Physiol* 1983; 245: C172-177). The change from white to brown fat is accompanied by the appearance of the typical uncoupling protein in the inner mitochondrial membrane. This observation have attracted the attention of pharmacologists that have tried to discover an analog of epinephrine, in particular a β 3-receptor selective agonist, that would cause excess dietary calories to be burned up and released as heat rather than stored as fat. Pharmaceutical companies have devoted enormous resources to this mechanism of action by means of agonists of the β 3-adrenergic receptor, without a single drug approved to date because of hypertension and cardiovascular effects due to the presence of this receptor in the heart. Burning fat occurs through mitochondrial oxidation and is performed by brown adipocytes (BA), not white adipocytes (WA). The key would be to screen for drugs that convert WA into BA without the adverse effects of the adrenergic agonists. Alternatively, we could screen for modulator drugs (see next paragraph) that as adjunct therapy potentiate the effect of the β 3-agonist, enabling lower doses of the β 3-agonist devoid of the adverse effects. This strategy has not been possible because there are only a small percentage of BA immersed within WA, and most screening technologies cannot detect these small %s. ExviTech is based on flow cytometry, a technique where cells are interrogated one by one at high resolution, commonly used for immunophenotyping, with the ability to accurately identify 1 in a million cells. Thus, the Exvitech platform is uniquely positioned to measure accurately these small % of BA versus WA, and thus to screen for the effect of any known drug in increasing significantly the small % of BAs.
4. **Efficiency. Modulating Therapeutic Agents:** This new technology allows Vivia Biotech to identify modulating agents dependent of the biological context in which they have to act since using fresh patient samples. We will observe cellular reactions of patients in the biological context in which the disease operates (excess fat in adipocytes), that is, the closest to the real biological milieu possible. These therapeutic agents will be prescribed as an adjunct therapy multiplying the effect of approved drugs reducing dosage and thus reducing toxicity. This advantage, applied to existing therapies, would, in addition, capitalize and reaffirm established markets for the existing therapies.
5. **Synergy between Therapeutic Agent – Personalized Medicine Test or Biomarker:** It is of great interest to the field to approve new treatments whose effectiveness has not been proved sufficiently for a specific indication associated with a biological test to identify those patients which will benefit most from the therapy. This type of test is called Personalized Medicine since they allow the therapy to be “personalized” to those patients who will benefit most. Another common denomination for this process is Pharmacogenomics, since the majority of these tests are use genomics to identify the best suited patient. Vivia Biotech test is cellular, and it is the same is been used in the screening of therapeutic agents, to evaluate its efficacy in reducing fat or glucose transport in abdominal adipocytes in a fresh sample. To be the first ones to test thousands of drugs in fresh adipocytes will allow us identify without additional efforts those possible treatments only working on a subgroup of patients could be approved associated with a Personalized Medicine test in the same sample. Biomarkers used to identify those patients and described below will also serve to better guide use of approved agents such as Rimonabant.

1.3 Description of the main S & T results/foregrounds.

Reprobesity was a project designed to search new therapies for complicated obesity, by using the technology of reprofiling. *Reprofiling* is the search of new indications of existing drugs. The project was oriented to improve the existent therapeutic options for the pharmacological treatment of obesity. However, along the first 12 months of execution, *Reprobesity Consortium* had to face a dramatic situation: the withdrawal of marketing authorisation of almost all drugs approved to treat obesity. That meant that the main aims of *Reprobesity* were severely affected by the fact that there were no standard protocols for the treatment of obesity to be used as reference standpoints. Practically, from its very beginning, *Reprobesity* was facing a situation where almost all pharmaceutical companies were losing interest in the development of obesity therapeutics. However, the extraordinary quality of the scientific teams involved in the project, and the solid design allowed the Consortium to reach the main goals proposed in the technical annex of the proposal. Thus, along the last 42 months, the *Reprobesity Consortium* has generated a significant amount of results that include:

- a) technological foreground, including utility models and technological patents,
- b) industrial patents for medicines designed to treat obesity and diabetes, and
- c) more than 50 scientific reports in peer-review journals, including top scientific journals such as Nature Neuroscience, Cell Metabolism, Journal of Neuroscience, Diabetes etc...

The description of the main scientific and technological results will follow the foreground table included in the present report (Point 2), and structured around the main objectives of the project.

1. Technological Foreground

Reprobesity Consortium was focused on the activity of the SME VIVIA Biotech, that had the objectives of using its Flow Cytometry platform ExVltech to develop two types of products:

- a) A new methodology for reprofiling existing drugs by analyzing the response of either, adipocytes or cell lines engineered to express targets for pharmaceutical development, to libraries of pharmaceutical compounds, and
- b) A new methodology for generating functional cytomic biomarkers that can be used in a combinatorial way to establish cellular fingerprints that serves as phenotyping landmarks in complicated obesity patients.

In addition, the other members, the academic partners of the *Consortium* developed two types of instruments, some of them patentable but others just late state of the art models for studying the biology of obesity. This foreground included:

- c) Tissue-specific genetically modified animals
- d) New chemical entities modelled upon existing drug that may serve as tools for further pharmaceutical developments.

A. The reprofiling technique

The objective is to identify new indications of existing drugs, alone or in combination, with potential anti-obesity efficacy by lowering the fat content and the glucose uptake of abdominal fat cells, which would be expected to

improve carbohydrate/lipid metabolism and lower body weight, extracted from the above phenotyped patients. This will be accomplished by screening *ex vivo* approximately 2.000 known drugs against adipocytes using the novel technology platform “ExviTech” from Vivia Biotech. The main objective of this deliverable is to set up functional assays for flow cytometry to measure lipolysis, thermogenesis, oxidation, and glucose uptake in freshly isolated abdominal fat adipocytes from animal models and patient samples.

Since adipocytes are the main cell clustered in the adipose tissue, we need first to digest the tissue and to establish the optimal conditions for culture. After extensive research, we focused the study in the analysis of adipocyte of less than 70 μm , obtained through filtration of crude digestion of abdominal adipose tissue. After collagenase incubation, samples are filtered through 70 μm filters, diluted in 12-36 ml of complete medium (DMEM, 10% FBS, P/S, L-Glu), and dispensed in 12 well plates. Around 1 million cells are dispensed per well, containing between 50.000 and 100.000 adipocytes. On these wells we search for three endpoints measured by flow cytometry:

- a) Amount of lipids per cell, cell morphology
- b) Total mitochondrial volume, mitochondrial membrane potential
- c) Fenton reaction to identify Brown adipocyte-like phenotype

These end points were achieved by the use of specific fluorochromes, including

- a) Bodipy, MFI and Side scatter properties for lipid content
- b) Mitotracker green, Rhodamine 123 and Tetramethylrhodaminemethylester for mitochondrial volume and mitochondrial membrane potential.
- c) For the evaluation of reactive oxygen species (ROS) on the Fenton reaction to identify peroxide formation for brown or brown-like adipocytes we used dihydrochloro fluorescein.

We discarded for the screening test the measurement of glucose uptake since it was found to be very quick and non-compatible with timing and spectral compatibility requirements for high throughput screening, although it will be used for confirmation and characterization of candidates.

After the incubation of the cultured cells with the fluorochromes we measured the specific fluorescences for each fluorochrome, both, in white adipose-derived and brown adipose-derived cells, in order to establish fully differentiated phenotypes.

In order to test the ability of detecting changes in white adipocytes towards brown-like adipocytes, we measured the specific response of white adipocyte to Adrenaline (0.1 to 100 μM), as a positive control for thermogenesis and oxidation, and compared it to an specific $\beta 3$ -adrenergic agonist (either Mirabegron or BRL 37344). The results were compared to the responses obtained on brown adipocytes. Finally, and as a control for the opposite phenotype, we tested the pro-lipogenic drug methylprednisolone.

The functional assay reveals that we can differentiate white and brown adipose cells on the basis of the selected parameters. Moreover, the response of white adipose cells to adrenaline, promotes a functional differentiation to brown-like phenotype of response, as shown in the following figures.

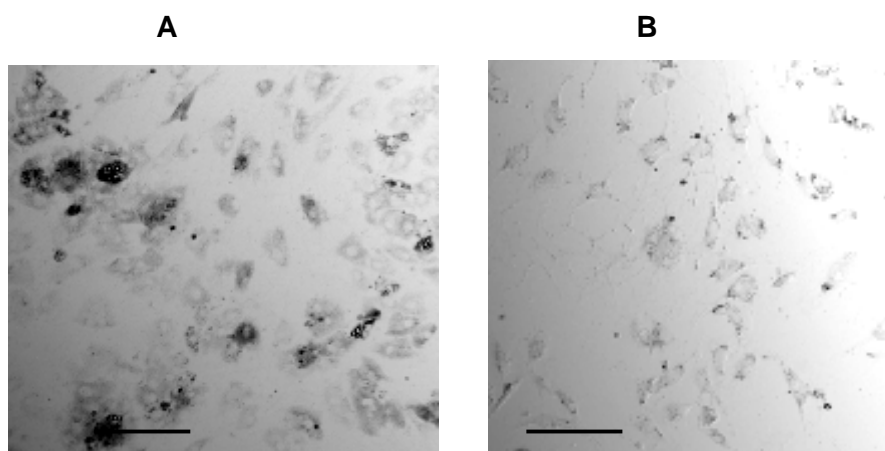


Figure 1.- Images of lipid contents as stained with Nile red of white adipocytes cultured in the absence (A) or presence (B) of adrenaline during 6 days,. Adrenaline decreases dramatically the content of lipid in cultured cells, which display small lipid droplets. Bar 100 μ m.

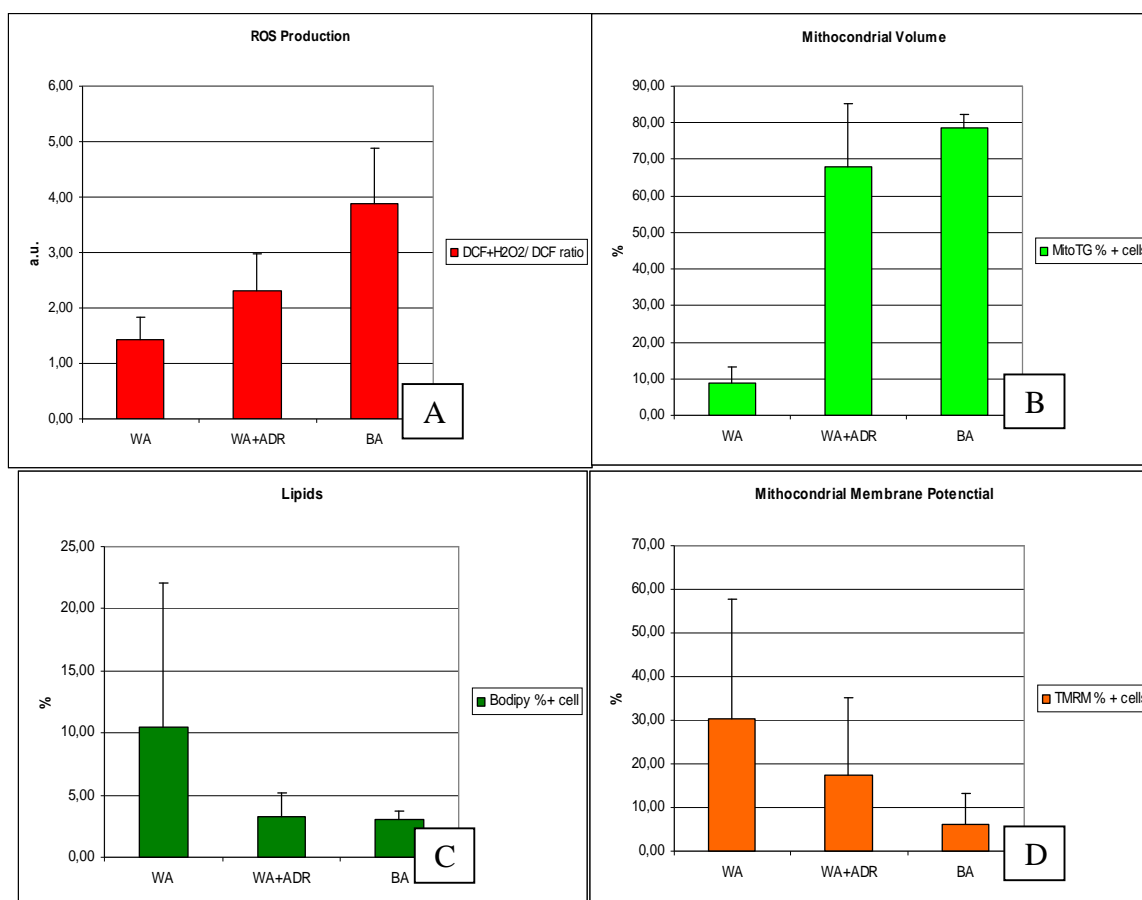


Figure 2. A. Brown adipocytes (BA) produced more ROS than white adipocytes (WA). The addition of adrenaline results in an increase in ROS production on WA that equals to that of normal BA. **B.** Mitochondrial volume of BA is higher than that of WA. Adrenaline induces an increase in mitochondrial volume on WA. **C.** BA has less lipid content than WA. Adrenaline lowers lipids in the WA. **D.** Mitochondrial membrane potential is lowered in thermogenic BA. Adrenaline reduces mitochondrial membrane potential in WA.

Similar results were obtained with β 3-adrenergic compounds tested, and the opposite with the prolipogenic drug methylprednisolone, as seen in next figure.

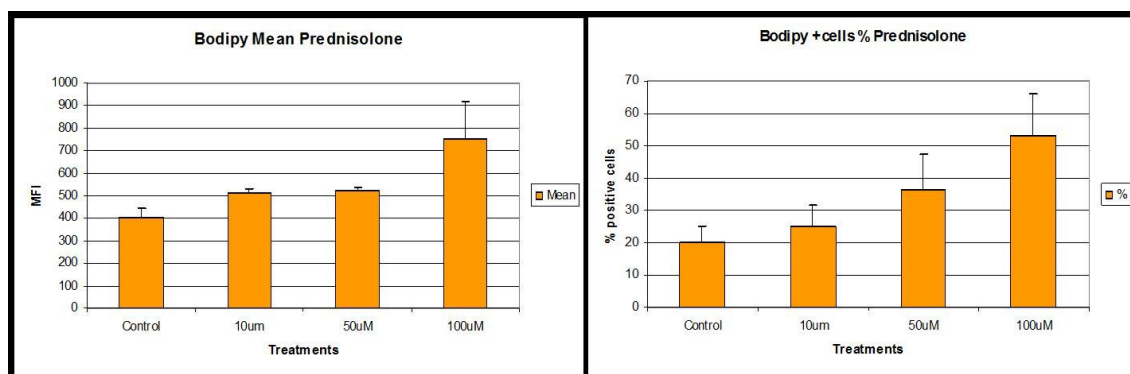


Figure 3. Culture of adipose tissue cells with the glucocorticoid methylprednisolone increases lipid content on white adipose cells in a concentration dependent manner.

Based on this assay, we screened the most relevant β 3-adrenergic receptor agonists. They included Mirabegron, CL31543, BRL37344, ICI215001, CGP12177 and ZD2079. These compounds were evaluated because they were eventually tested in clinical trials in obesity or neurogenic bladder. They were screened alone or in combination with three main classes of drugs:

- GLP-1 receptor agonists, since GLP-1 receptor controls lipolysis in adipose cells (Endocrinology. 2011 Nov; 152(11):4072-9).
- PPARalpha agonists, focusing on its endogenous ligand oleoylethanolamide (OEA). This receptor is involved in fatty acid oxidation as well as in the control of thermogenic responses. The consortium has also developed OEA-based analogues that resulted in activation of thermogenic responses (Dis Model Mech. 2012 Jun 26.)
- The cannabinoid receptor antagonist Rimonabant, since the CB1 receptor controls lipolysis, mitochondriogenesis and thermogenic responses in adipose tissue (Diabetes. 2010 Nov;59(11):2826-36)

From screening results the most active compound found was CL31543, as well as the combination with either, Liraglutide (a GLP-1 receptor peptidic agonist) or OEA (the endogenous ligand for PPARalpha receptor and also a GPR119 agonist). In order to verify the selection of candidates we performed *in vivo* studies to demonstrate that a) CL31543 alone or in combination with OEA or Liraglutide reduced feeding and b) Its chronic administration resulted in changes in fat mass. Next figures depict how the β 3-adrenergic agonist CL31543 when combined with either Liraglutide or OEA reduced fat mass, by inducing increases in thermogenesis and changes in energy expenditure.

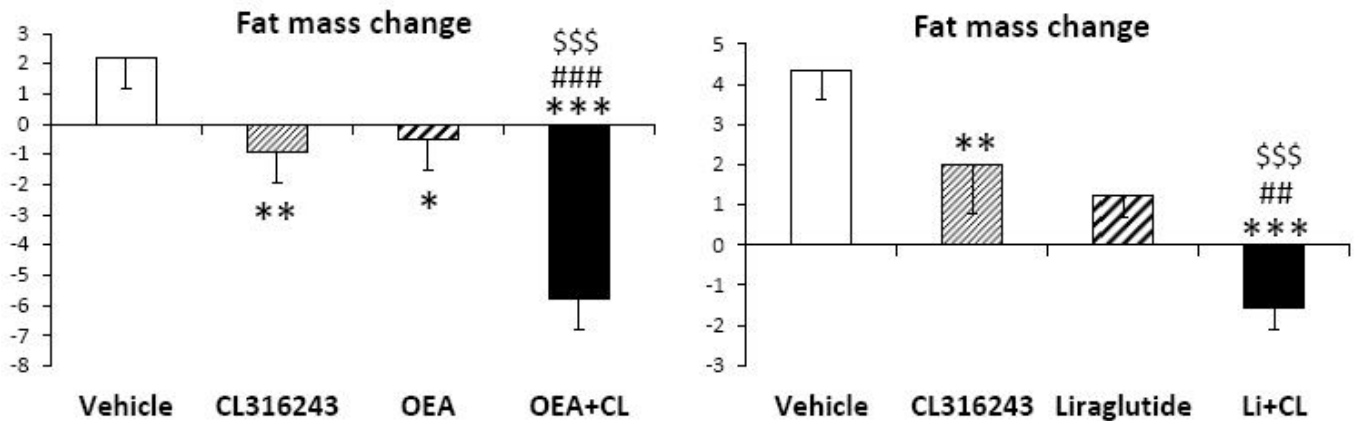


Figure 4. Net fat mass reduction after subchronic administration of the β_3 -adrenergic receptor agonist CL31543 combined with either OEA or Liraglutide in Wistar rats.

B. The Combinatorial Cytomic Biomarkers

VIVIA Biotech has applied its technologic platform ExviTech to measure response to therapeutic agents in blood biomarkers in a novel combined way. Once patients are well phenotyped clinically it is important to measure as many variables as possible in an unbiased way to identify those who correlate with clinical phenotypes which define the biomarkers. VIVIA has developed the Combinatorial Cytomic Biomarkers which will incorporate a great number of biological variables. To illustrate this point let's consider measuring with ExviTech the effect of 2000 agents in whole blood samples distinguishing five types of cells and measuring 30 biological indicators for cell. The result is $2000 \times 5 \times 30 = 300.000$ possible cellular biomarkers. The screen performed followed two different modes: one for kinetic biomarkers, which require real-time assays on the order of few seconds after mixing the drug with the cells, and another for end point assay biomarkers, that measure biological parameters after hours of incubation with the drug, typically approximately 24 hours that means the next day. Typical kinetic assays are ion concentrations such as calcium and sodium. Typical end point assays are the lypolysis, thermogenesis, and glucose uptake assays described above for the reprofiling effort, which would be used here as biological indicators. For both kinetic and end point assays, the key is to maximize the multiplexing capacity to measure simultaneously in every cell as many different assays as possible. This means mixing with the same sample as many fluorochromes as possible that measure a desired biological parameter, as long as their fluorescence emissions are distinguishable.

Using standard fluorochromes we can mix between 5-10 fluorochromes and measure them simultaneously. We will thus group fluorochromes based on two guiding principles: that they measure different parameters of the same pathway (e.g. oxidation, ion fluxes, lipid content, immune system, glucose system, platelet aggregation, etc...), and that they are spectrally compatible, necessary to distinguish their signals after data acquisition. The set of how many different cell types we can distinguish within a sample also determines the number of biomarkers measured, due to the combinatorial effect (compounds \times cell types \times assays). For blood samples, we expect to distinguish about 5 cell types with significant presence to enable statistical data acquisition. For abdominal adipose tissue samples, treated with collagenase to generate single cell suspensions, we also expect several cell types, although we haven't been able to quantify their %s yet. In addition to white adipocytes, the most common cell

type, we observe also brown adipocytes that are extremely important for thermogenesis, as well as macrophages and T-Cells.

The set of compounds with diverse biological activities to screen against the ex vivo samples to generate the Combinatorial Cytomic Biomarkers (CCB) are actually the same set of known drugs used above for reprofiling. The selection of known drugs has been made precisely based on representing a wide spectrum of activities, which is what we need to use for biomarkers. Because the assays for reprofiling are assays utilized here as well as biomarkers, the screening for Reprofiling represents the first set of CCBs to be measured, and that effort is synergistic with the effort to discover biomarkers of obesity.

The functional response of blood cells is very sensitive, and we will always use samples extracted first thing in the morning while still fasting. In addition to the steps described above, we should first identify in control blood samples those assays among all available to flow cytometry that show a limited day-to-day variation. This will be accomplished by measuring all available fluorochromes in control blood samples of individuals at differing times, ideally on a bi-weekly basis for 3 months.

Results

CCBs on the Pizarra cohort

Samples from the Pizarra study (A cohort following nutritional habits of 1500 inhabitants of a typical Andalusian location) were used for monitoring both:

- a) the response to a selected set of pro-oxidants/antioxidants compounds, (See next figure) and
- b) the response to a selection of 80 drugs interacting with targets present in leukocytes.

In both cases, the raw fluorescence data were imported into SPSS and merged with the epidemiological data collected with each sample, allowing a multivariate analysis on which to test the hypothesis of the phenotypic characterization of ROS production in obesity.

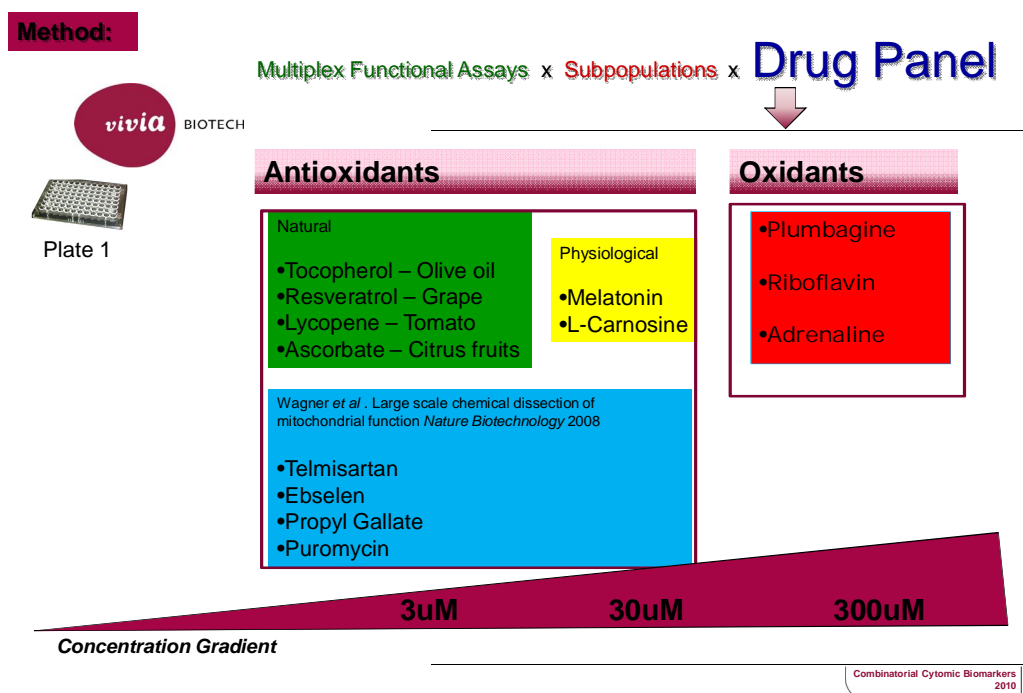


Figure 5. Drugs selected for the antioxidant plates.

A total of 74 cases were finally included for analysis. As depicted in the next tables, Pizarra volunteers exhibited a high prevalence of obesity, as reflected in BMI distribution

Table 1. BMI categories in the Pizarra cohort samples

Sex	BMI Categories			Total
	Normal	Overweight	Obese	
F	7	19	15	41
M	4	11	18	33
Total	11 (15%)	30 (40.5%)	33 (44%)	74

On these cases, basal and drug-modulated ROS production, but not thiols concentration, was found to be different on the categories of Normal versus Obese patients.

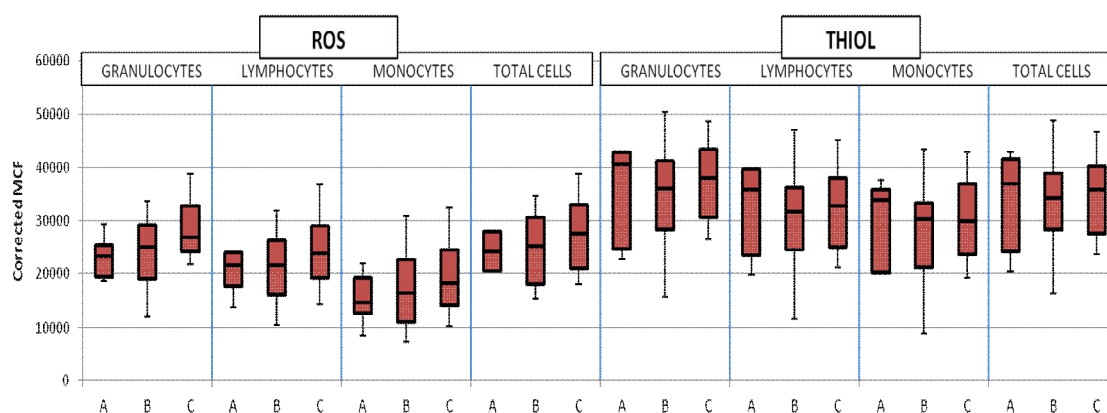


Figure 6. Basal ROS and Thiol levels regarding three categories of BMI (Normal (A), Overweight (B) and Obese (C)).

In fact, the overweight group always stand in between both extremes of the phenotypes. When the response to different antioxidants/pro-oxidants was analysed, a similar pattern of results was found. (see next figure). This indicates that the actual technology allows the identification of obese phenotypes just because the presence of a specific pattern of ROS production.

In order to fully address this hypothesis, it was performed a cluster analysis with the raw fluorescence. Cluster analysis group cases on the basis of similarities on the variables under study. As it can be seen below, a hierarchical cluster analysis based on Ward's method of minimal variance was done for the responses found on granulocytes at 3uM using the MCF signal from ROS marker.

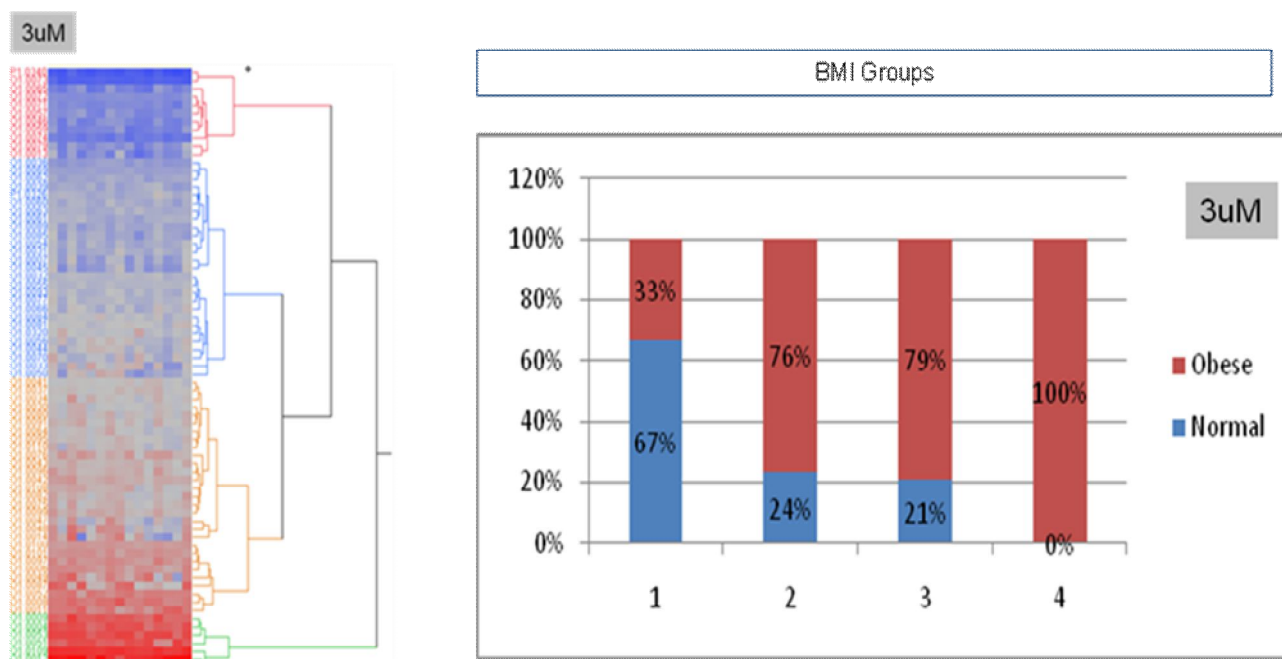


Figure 7. Cluster analysis of ROS levels after exposure to different pro-oxidants and anti-oxidants drugs, in the different IDF clusters.

This analysis indicates clearly that the ROS response is useful to differentiate between groups of obese people that exhibit a clearly enhanced ROS response associated with less efficacy of antioxidants, and normal people where a majority exhibit a normal ROS response. Overweight people are distributed along all the clusters, indicating that some of them behave as normal whereas other overweight behaves as the obese. Finally, a multivariate analysis performed to this sample indicates that the ROS response is a composite response of factor including mean systolic blood pressure, glycaemia, cholesterol, triglycerides, body mass index, urea and creatinine values, indicating that this type of biomarker ROS analysis may help to establish a cluster of people whose risk factors will determine the health status and future quality of life.

CCBs in the Massa Lombarda Cohort

Along the first reporting period, we developed the concept of Combinatorial Cytomic Biomarkers and implemented it to the analysis of oxidative stress in population, as well as to the in vitro analysis of the antioxidant potential on natural products and commercial drugs. In the second periodic report we have developed a different technology where we test the hypothesis that the acquired phenotype of leukocytes, as results of genetics x environmental interactions, can be reflected in the activated metabolic profile of these cells. We developed the method so it can use frozen lymphocytes, using the standard procedures for leukocyte freezing in the blood biobanks. The technology is based on the activation of immune cells by two different stimuli, proliferative stimuli (i.e. incubation with bacterial endotoxin B, LPS and antibody against aCD28), metabolic stimuli (Insulin) or the combination of both. In the stimulated cells, we will measure the translocation to the surface, or the internalization of a) The GLUT1 glucose transporter, b) The insulin receptor and c) The fatty acid transporter/scavenger receptor CD36. Using flow cytometry properties of immune cells, as well as labelled antibodies against surface receptors we can differentiate a total of 19 different resting/activated populations where to measure these three metabolically relevant proteins.

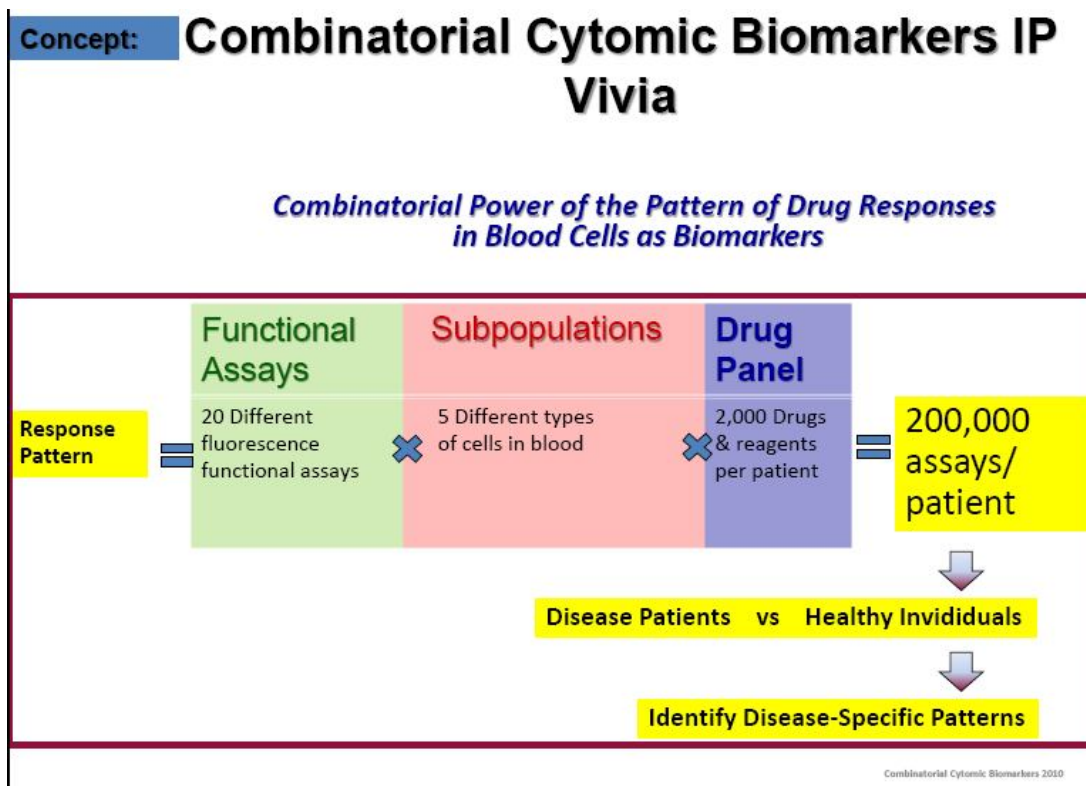


Figure 8. *The Concept of Combinatorial Cytomic Biomarkers.*

After setting up the multiplexed assay on human lymphocytes, a selected population of extreme phenotypes of the Masa-Lombarda population was chosen for evaluating functional CCBs. The characteristics of this population of extreme phenotypes were as follows:

The extreme phenotypes selected were classified as normal or “metabolic syndrome” using this terminology for depicting obese people with cardiovascular risk factors including elevates waist circumference, BMI around 30, high systolic pressure, high circulating cholesterol and triglycerides and hyperinsulinemia. Blood from these subjects was withdrawn and cells separated and used as described in WP4. After stimulation with immune/insulin stimulus and flow cytometry analysis of the expression of surface markers (Glucose transporter 1, CD36 and Insulin receptor), the functional data analysis yield the following results:

- Main changes in between both phenotypes, regarding glucose transporter expression occurred in activated T cells, whereas B cells or monocytes contributed less to the changes observed after stimulus.
- There were no changes in CD36 expression in both extreme phenotypes.
- Differences in proliferation responses were observed only when the stimulus was insulin.

DESCRIPTION OF EXTREME PHENOTYPES ASSAYED MASSA-LOMBARDA STUDY

PARAMETER	HEALTHY	METABOLIC SYNDROME
TOTAL CASES	36	41
MALES	10	17
FEMALES	26	24
AGE	40 ± 2	55 ± 2
BODY WEIGHT	59 ± 1	83 ± 2
BMI	21.4 ± 0.3	29.7 ± 0.6
WAIST CIRC (cm)	73.9 ± 1.1	100.6 ± 1.7
HIP CIRC (cm)	93.8 ± 1.2	110.2 ± 1.4
SBP (mm Hg)	112.2 ± 1.2	133.2 ± 3.5
DBP (mm Hg)	74.1 ± 1.1	83.4 ± 1.3
TRIGLYCERIDES (mg/dl)	62.9 ± 4.3	150.2 ± 11.8
CHOLESTEROL (mg/dl)	171.2 ± 4.4	205.4 ± 5.8
HDL (mg/dl)	62 ± 1.8	44.1 ± 1.7
INSULIN (uU/ml)	4.9 ± 0.4	11.6 ± 1.5
GLYCAEMIA (mg/dl)	84.4 ± 1.4	104.3 ± 4.5
URIC (mg/dl)	4.1 ± 0.2	5.2 ± 0.2

Table 2. Extreme phenotypes of Massa-Lombarda subjects used for the functional evaluation of CCBs.

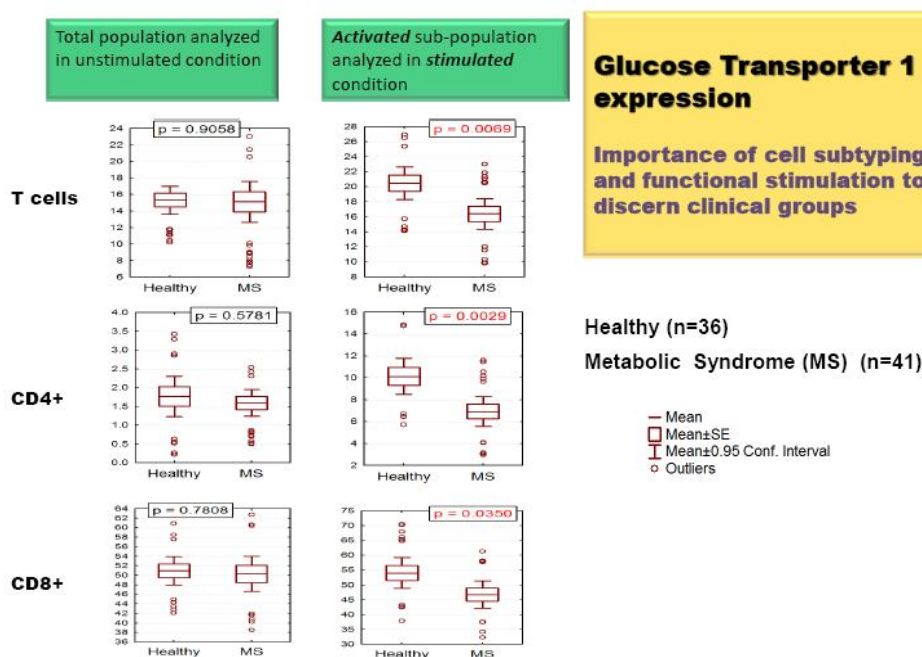


Figure 9. Changes in mean intensity of fluorescence for glucose transporter 1 (GLUT1) in between extreme phenotypes of the Massa-Lombarda population. Most differences were observed in activated T cells. In all cases, metabolic syndrome is associated to a decrease in the expression of the transporter.

Populational studies such as that of Pizarra or Masa-Lombarda allows to monitor and contrast experimental hypothesis without the costs and difficulties of a clinical trial or a recruitment of hospitalized patients. The Pizarra study has the advantage of being focused on nutritional and life style factors determining metabolic disorders including obesity and diabetes. The data presented here showed that by measuring the ability of pro-oxidant or anti-oxidant compounds to modulate ROS production in leukocytes, we can differentiate obese from normal population. This differentiation is clear and surprisingly showed that overweight people (i.e. BMI less than 30) displays an erratic behaviour. This finding is very relevant because the real situation is that not all the overweighted people are ill, so the responses of a percentage of them may be similar to that of healthy volunteers (healthy overweight-healthy obese population). Additional and wider studies are needed in order to catalogue the real impact of this new approach on setting risk factor for cardiovascular disorders on the basis of a cytoxic biomarker.

Regarding metabolic biomarkers, the results indicate that this approach allows the identification of differential responses of lymphocytes to activatory stimulus such as immune challenges (i.e. lps, enterotoxin b) and/or insulin. Interestingly, a clear division can be observed in between people suffering metabolic syndrome and healthy subjects. The differences can be magnified either by the type of the stimulus or the subtype of cell population studied. In general, Lymphocytes T exhibited a better differential response. Thus, stimulus revealed a lower expression of glucose transporter 1 GLUT-1, lower expression of insulin receptor and lower proliferation indexes. Multivariate analysis of variance is being now addressed in order to unveil the association of these types of responses with the clinical phenotypes.

C. The tissue specific genetically modified animals for studying obesity

During the reporting period the consortium has focused on target validation and development of in vivo genetically modified animal models for the explanation of:

1. The peripheral action of cannabinoid receptor ligands by designing cannabinoid CB1 receptor knockout mice lacking the receptor in either the sympathetic neurons or the adipose tissue.
2. The central actions of endocannabinoids for modulating appetite by addressing the actions in different cannabinoid receptor knockout mice expressed by subsets of neurons.
3. The validation of 1,2,4,oxadiazol derivatives candidates in GLP-1 receptor knockout mice.

The peripheral action of cannabinoid receptor ligands.

In collaborative work partners 3, 5 and 7, a central role of CB1 receptor in adiposity and regulation of energy homeostasis was assigned to neuronally-expressed CB1 receptor. It was shown that the sympathetic outflow is increased in mice specifically lacking neuronal CB1 receptor and is responsible for the lean phenotype under high fat diet. The research has focussed mainly on the role of the endocannabinoid system in regulating energy expenditure and adiposity at the level of sympathetic neurons and adipocytes. We believe that exploring the peripheral mechanisms of endocannabinoid-mediated regulation of adiposity is very promising in the perspective of therapeutic approaches using peripherally restricted blockade of CB1 receptors.

The collaborative study has pursued the following projects:

- (A) Generation and breeding of CB1 receptor knockout mice specific to adipocytes and sympathetic neurons.
- (B) To evaluate metabolic features and mechanisms regulating energy homeostasis in mice lacking CB1 receptor in adipocytes and sympathetic neurons.

- (C) To establish an in vitro culture system for sympathetic neurons and adipocytes and their co-culture for the evaluation of therapeutic drugs.
- (D) Role of CB1 receptors expressed in brain and sympathetic neurons.

(A) Breeding of conditional CB1 knockout mice

dbh-CB1-KO: During the first year, we generated CB1 receptor knockout mice specific for the sympathetic neurons. We crossed CB1 floxed mice with mice expressing cre recombinase under the regulatory sequences of dopamine β hydroxylase gene (dbh-cre) in order to knockout CB1 receptor from the dbh expressing neurons only. This breeding was successful and we obtained a sufficient number of conditional knock-outs for first analyses. This mouse line is named dbh-CB1-KO and the wild-type littermate dbh-CB1-WT.

ati-CB1-KO: Secondly, we have generated adipocyte-specific CB1 knock-outs, as adipocytes play a key role in controlling lipid metabolism and energy homeostasis. In fact, CB1 receptors were shown to have important roles in adipocyte physiology, and their expression is dramatically increased during obesity. We think that this new mouse line lacking the CB1 expression in adipocytes will represent another key model to test and validate new anti-obesity drugs. To this end, we used an inducible Cre/loxP system, which enables us to delete CB1 receptor expression in adipocytes. This line was named ati-Cre-KO mice. To confer cell-type specificity in recombination, the cre recombinase is under the regulatory sequences of the adiponectin gene. To be able to confer temporal specificity of the activity of the Cre recombinase, the protein was fused to a modified estrogen-binding domain (Cre^{ERT2}) lacking its binding activity for the endogenous estrogen 17 β -oestradiol, but still being able to recognize the anti-oestrogen drug 4-hydroxy-tamoxifen (also called: Tamoxifen). When Cre^{ERT2} is present in absence of Tamoxifen, it remains in the cytoplasm and only when the drug is applied, the fusion protein translocates into the nucleus and mediates the recombination, and thus, deletion of the CB1 receptor gene.

Mice were injected with 1 mg tamoxifen/day for 5 days. Food intake and body weight are recorded daily; animals had free access to food and water. After three weeks, during which CB1 gene deletion was completed and the remaining CB1 receptor proteins in adipocytes were removed, mice were put into different groups receiving SD- or HF-diet for a period of 12 weeks.

Metabolic characterization

Glucose metabolism of dbh-CB1-KO

The brain integrates inputs from peripheral organs and blood (glucose, free fatty acid, hormones) and mediates appropriate outputs to different peripheral organs in order to regulate energy homeostasis. Sympathetic and parasympathetic neurons innervate different peripheral organs such as pancreas, liver, adrenal gland, brown adipose tissue (BAT), white adipose tissue (WAT) etc. to regulate metabolism and energy homeostasis. Increased sympathetic outflow not only increases thermogenesis in BAT, but also glucose metabolism in liver, epinephrine release from adrenal gland and glucagon release from pancreas. Therefore, deleting CB1 receptor from sympathetic neurons may affect the overall energy homeostasis and metabolism. We are in the process of characterizing dbh-CB1-KO mice for their metabolic phenotypes. We monitored glucose tolerance and insulin tolerance after starvation for 24 hour, and blood glucose over the course of the 24-hour starvation period. Our results suggest that dbh-CB1-KO have a tendency to show less glucose tolerance than the WT control mice when challenged in the intraperitoneal glucose tolerance test (IPGTT). Moreover, the insulin tolerance test (ITT) showed that dbh-CB1-KO mice can protect from lowering their blood glucose levels in much more efficiently than WT controls,

suggesting that the counter regulatory mechanism mediated by glucagon and/or epinephrine release is possibly affected. Glucagon and epinephrine, secreted after sympathetic stimulation of pancreas and adrenal gland, respectively, increases glycogen breakdown and activity of enzymes involved in gluconeogenic pathway in order to protect from a drop in blood glucose level. dbh-CB1-KO mice were able to protect fasting-induced reduction in blood glucose in more efficiently than the WT controls. This suggests that the increased sympathetic activity and/or increased secretion of epinephrine and norepinephrine might be responsible for the higher blood glucose during GTT and starvation. Type 2 diabetic patients are known to have abnormal glucagon secretion and generally show higher glucagon level, and therefore, exhibit increased glycogenolysis.

ati-CB₁-KO mice are resistant to develop diet induced obesity and related metabolic impairments:

after tamoxifen treatment, mice were sorted into different groups receiving standard or high fat diet (SD and HFD, respectively). Body weight and food intake were recorded twice a week. Ati-CB₁-KO mice showed a reduced body weight due to a reduction of total body adiposity and a reduced food intake, as compared to their wild type littermates, independently from the diet treatment (Fig. 10 a-c)

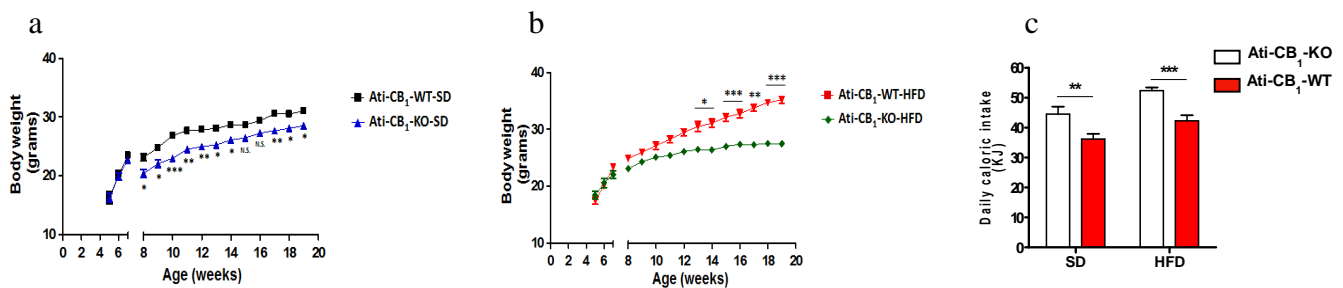


Figure 10: (A) Weight evolution in *ati-CB1*-WT. vs. -KO mice in SD (B) and HFD. (C) Daily caloric intake in both phenotypes fed with either diet ** $p < 0.001$ Two way ANOVA test, Bonferroni post test.

CB₁ deletion in adipocytes from epididimal fat (EF) resulted in impaired differentiation and reduced lipid accumulation, in the subcutaneous fat (SF) to a white-to-brown phenotypic transition and in the mesenteric fat to an up-regulation of adiponectin synthesis and release. In order to verify whether the adipocyte-CB₁ deletion is able to reverse a condition of pre-existing obesity, CB₁ gene deletion has been induced after the onset of obesity in 16 weeks old animals kept on super-HFD (SHFD) and SD animals (mutants were named Ati-CB₁-KO_{TAO}, TAO: tamoxifen after obesity). As shown in Fig. 11, CB₁ gene deletion rapidly and strongly reduced body weight in HFD -fed Ati-CB₁-KO_{TAO} mice, to reach levels very similar as in SD animals. The reduction of body weight in SHFD -fed mutant mice was associated with a decreased amount of total EF and SF fat (Fig. 11b). Thus, adipocyte-CB₁ exerts a specific function under super-HFD conditions.

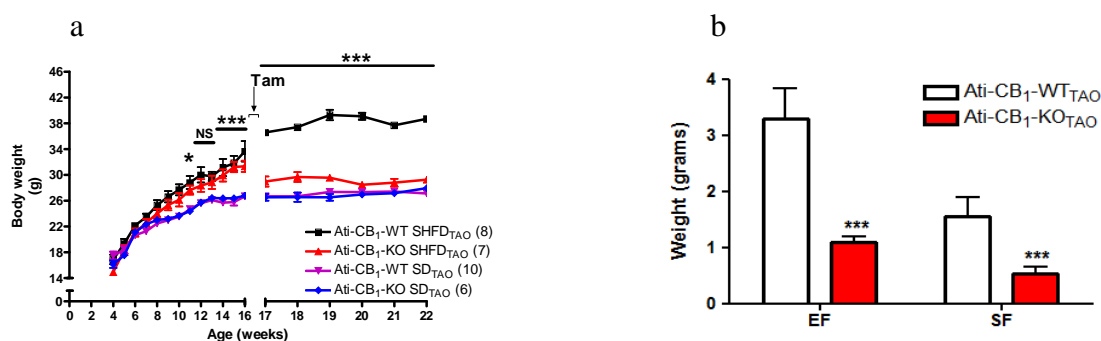


Figure 11: (A) Body weight gain in obese animals (Fed with superhigh fat diet SHFD) on which the deletion of the CB₁ receptor was induced in the adult age. (B) Evolution of the epididymal fat (EF) and the subcutaneous fat in *ati-CB₁-WT*. vs. *-KO* mice fed with SHFD after the activation of the CB₁ deletion. **p*<0.01 Two way ANOVA test, Bonferroni post test.

Our study demonstrates that adipocyte-CB₁ has a crucial role in regulating adipocyte physiology and body energy homeostasis. Besides, we demonstrated that targeting adipocyte-CB₁ might represent an extremely effective option for the treatment of obesity and its associated metabolic disorders.

D. The new chemical entities

An additional foreground was the synthesis of new chemical entities modelled upon existing drugs or endogenous signals regulating metabolism and energy expenditure, such as the acylethanolamides. Several of these molecules were developed and tested and some of them were patented, so their activity and utility is fully described in the following section and the corresponding patent applications. However, some of them were not patentable because they were based in formerly patented scaffolds. These tools, especially those that resemble existing drugs (pyrazols and triazols with a structure similar to that of the cannabinoid receptor antagonist Rimonabant and with a low permeability through the blood brain barrier) served as complementary instruments to confirm the hypothesis of the importance of peripheral cannabinoid CB₁ receptors in the regulation of metabolism, body weight gain and energy expenditure (See for example the study on the triazol LH-21, a cannabinoid receptor antagonist that does not cross the blood brain barrier, Alonso et al, Br J Pharmacol. 2012 Apr;165(7):2274-91).

Similar studies have been performed using oleoylethanolamide-based compounds with the capability of increasing energy expenditure, reducing body weight gain and promoting thermogenesis through the activation of the PPAR α receptor. Such is the case of elaidylsulfamide, an acyclic acylsulfamide with the capability of activating PPAR α receptors (Decara et al., Dis Model Mech. 2012 Jun 26.).

2. Industrial patents of medicines to treat obesity.

Table 3. Nine industrial patents have been filed by the members of Reprobesity Consortium.

	PATENT CODE	PATENT TITLE	INSTITUTIONS
1	P200930120 (Spain) PCT/ES2010/070293 (International) WO2010128191 (A1) (Publication)	BIVALENT PYRAZOLE DERIVATIVES AS FOOD INTAKE INHIBITORS	IMABIS, CSIC
2	PatentsP200931269 (Spain) PCT/ES2010/070854 (International) WO2011076966 (A1) (Publication)	FATTY ACID AMIDE DERIVATIVES WITH AMPHETAMINES FOR THE TREATMENT OF EATING DISORDERS	IMIM,CSIC,IMABIS
3	P201031878 (Spain) PCT/ES2011/070880 (International) WO2012080555 (A1) (Publication)	HYDROXYTYROSOL ETHERS	IMIM,CSIC,IMABIS
4	PCT/ES2011/070042 (International)	1,2,4-OXADIAZOLE DERIVATIVES AS MODULATOR DRUGS OF THE GLP-1 PEPTIDE RECEPTOR	IMABIS LICENSED TO VIVIA
5	P201130250 (Spain)	Uso de inhibidores de p53 para reducir la ingesta y para reducir el peso corporal	USC
6	P201131311 (Spain)	METODO PARA INHIBIR EL APETITO	Universidad de Santiago de Compostela; University of Aberdeen
7	PCT/ES2012/070276 (International)	Combination therapy for the treatment of metabolic diseases	FUNDACIÓN IMABIS; VIVIABIOTECH, S.L.; UNIVERSIDADE DE SANTIAGO DE COMPOSTELA; INSERM; JOHANNES GUTENBERG UNIVERSITAET MAINZ; ALMA MATER STUDIORUM-UNIVERSITA DI BOLOGNA
8	EP 12 005 364.0	Allosteric modulators of GLP-1	VIVIABIOTECH,S.L.; UNIVERSIDAD DE SANTIAGO DE COMPOSTELA; UNIVERSIDAD DE EXTREMADURA
9	EP 12 005 365.7	Allosteric modulators of GLP-1	VIVIABIOTECH,S.L.; UNIVERSIDAD DE SANTIAGO DE COMPOSTELA; UNIVERSIDAD DE EXTREMADURA

These industrial patents represent a great added value of the Consortium and gave the potentiality of selecting several patented strategies for placing a drug or a combination of drugs into clinical trials. We will focus here only in the two main candidates:

- The 1,2,4-oxadiazols as allosteric modulators of GLP-1 receptors
- The combinational therapy based in β 3-adrenergic receptor agonist combined with either OEA (PPAR α receptor agonist) or Liraglutide (GLP-1 receptor agonist).

The 1,2,4-oxadiazols as allosteric modulators of GLP-1 receptors

Main properties of the compounds identified as candidates are listed below, and some examples included in the following figures:

Properties:

- 1,2 4-oxadiazole derivatives were allosteric potentiators of GLP-1 agonists acting at GLP-1 receptors, as screened in several types of cells.
- They produce potentiation of the incretin response (Increase of Insulin secretion mediated by GLP-1 under high glucose).
- They are racemic mixtures and some of the enantiomers retain the ability to produce allosteric potentiation of the GLP-1 receptor.
- They produce potentiation of feeding inhibition mediated by GLP-1 agonists.
- They exhibited a good DMPK profile, making them suitable to progress towards clinical trials.

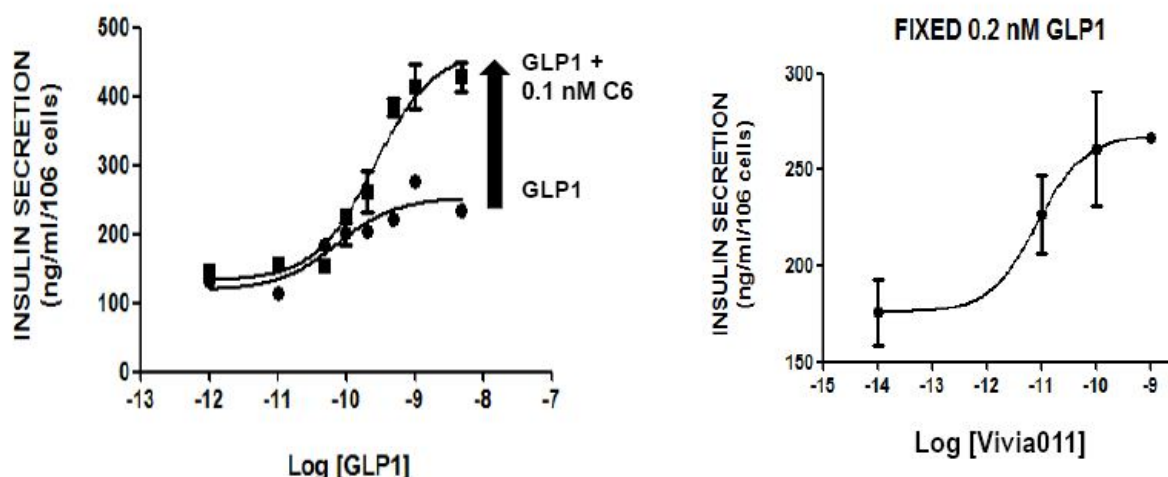


Figure 12. Effects of VIVIA011 as allosteric potentiator of GLP-1 receptor-mediated responses. VIVIA011 potentiates the response of INS1E insulinoma cells to GLP-1 stimulation under high glucose (15 mM) conditions.

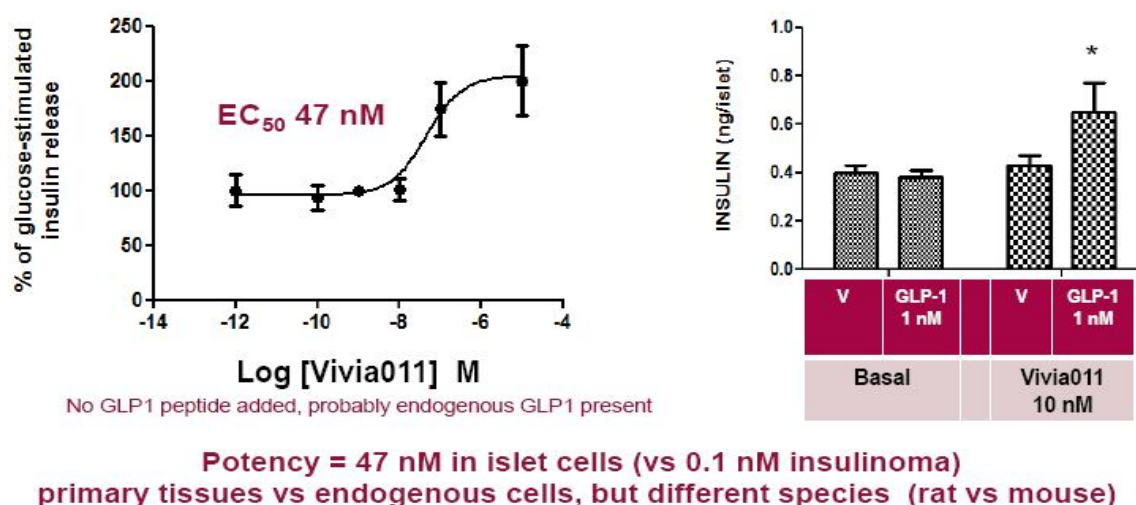


Figure 13. Effects of VIVIA011 as allosteric potentiator of GLP-1 receptor-mediated responses. VIVIA 011 potentiates the insulin secretion mediated by GLP-1 in mouse pancreatic islets.

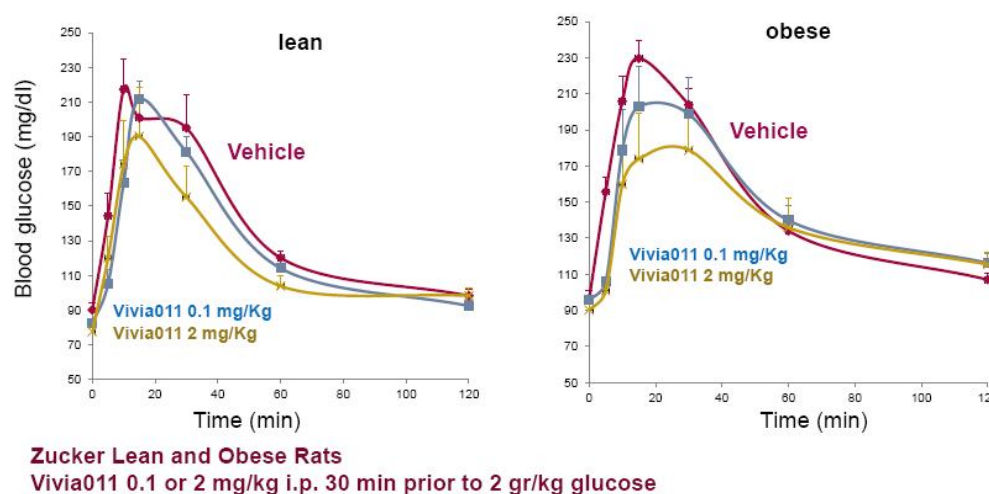


Figure 14. Effects of VIVIA011 as allosteric potentiator of GLP-1 receptor-mediated responses. VIVIA 011 improves glycaemia after an intraperitoneal glucose tolerance test, basically improving phase 1 of insulin release in obese and diabetic Zucker rats.

Once characterized in vivo and in vitro for its potential role as allosteric modulators of GLP-1 receptors, we characterized the actions of these compounds on feeding behaviour, both by central and peripheral administration. The present inventive allosteric compounds potentiate the effect of exendin-4 to the point where they become efficacious as anti-obesity drugs. Major efforts have been under way to show efficacy of these peptidic drugs in weight loss fight against a necessary significant increase in dosing, which may compromise their safety profile for long term treatment. The present inventive compounds potentiate these peptidic drugs to be efficacious in weight loss at tolerable doses, which means an important benefit for obese patients. In particular, there is a large co-morbidity between diabetes and obesity and thus such a combination treatment will have a major impact in patients' health and wellbeing. Obesity efficacy has been tested in animal models using an initial fasting model: 24 hour food-deprived rats. Thus, an intraperitoneal administration of compound VIVIA011 combined with a sub-effective dose of exendin-4

reduces food intake in food deprived Wistar. The therapeutic benefit of a combination of the present allosteric potentiators of the GLP-1 receptor and DPP IV inhibitors can be predicted on the basis of their respective mechanisms of action. DPP IV inhibitors inhibit the protease that cleaves endogenous GLP-1, DPP IV, resulting in higher levels of endogenous GLP-1 peptide. This additional GLP-1 peptide would be expected to be equally potentiated by the present GLP-1 allosteric potentiators. Such potentiation will enable a high efficacy combination of small molecule oral drugs, cost-effective with future generic DPP IV, with potential benefits both in diabetes and obesity.

In the next figures we show how the reference compound VIVIA011 is capable of potentiating feeding inhibition mediated by GLP-1 receptor agonists, when given through a central or a peripheral route. We will also show how this compound improves glycaemia in a glucose tolerance test through GLP-1 receptors, since this effect is not present in GLP-1 receptor KO mice. We will also show how one enantiomer of this compound displays great activity in the human receptor controlling insulin release.

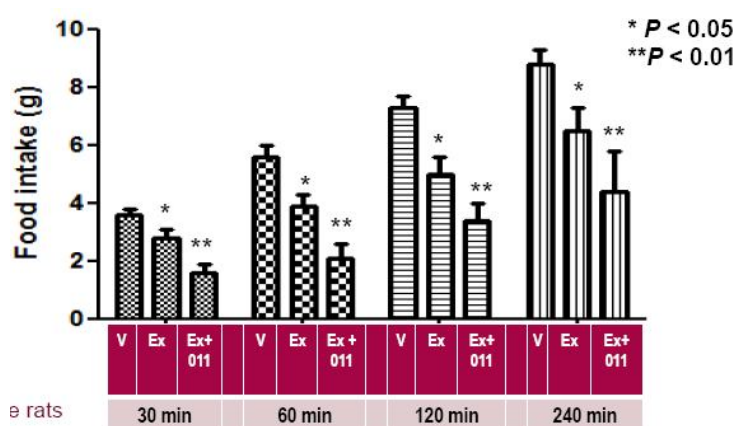


Figure 15. Effects of VIVIA011 as allosteric potentiator of GLP-1 receptor-mediated responses. VIVIA 011 potentiates the feeding inhibition mediated by the GLP-1 receptor agonist EXENDIN 4 when injected intracerebroventricularly in Wistar rats deprived of food for 24 h.

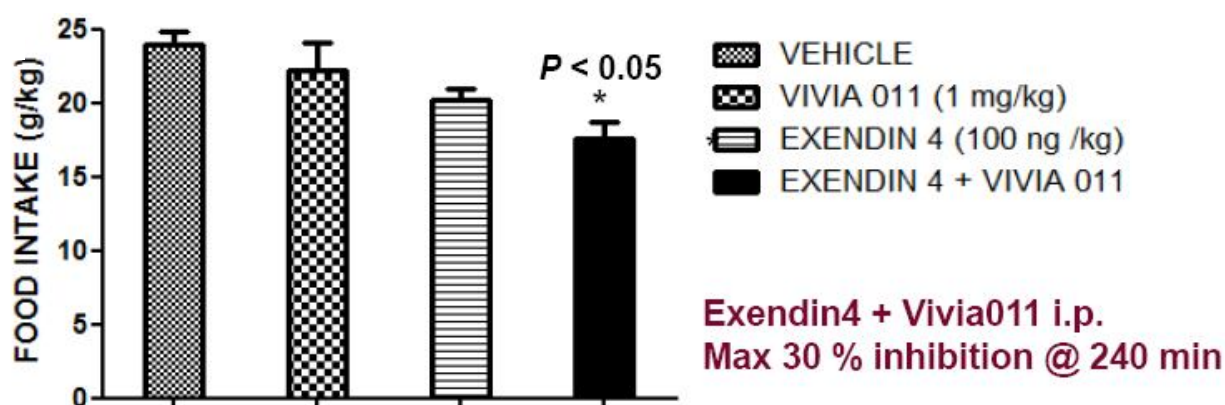


Figure 16. Effects of VIVIA011 as allosteric potentiator of GLP-1 receptor-mediated responses. VIVIA 011 potentiates the feeding inhibition mediated by the GLP-1 receptor agonist EXENDIN 4 when injected peripherally in Wistar rats deprived of food for 24 h.

The combinational therapy based in $\beta 3$ -adrenergic receptor agonist combined with either OEA (PPAR α receptor agonist) or Liraglutide (GLP-1 receptor agonist).

From screening results the most active compound found was CL31543, as well as the combination with either, Liraglutide (A GLP-1 receptor peptidic agonist) or OEA (the endogenous ligand for PPAR α receptor and also a GPR119 agonist). In order to verify the selection of candidates we performed *in vivo* studies to demonstrate that a) CL31543 alone or in combination with OEA or Liraglutide reduced feeding and b) Its chronic administration resulted in changes in fat mass. Next figures depict how the $\beta 3$ -adrenergic agonist CL31543 reduced feeding and combined with either Liraglutide or OEA reduced fat mass, by inducing increases in thermogenesis and changes in energy expenditure:

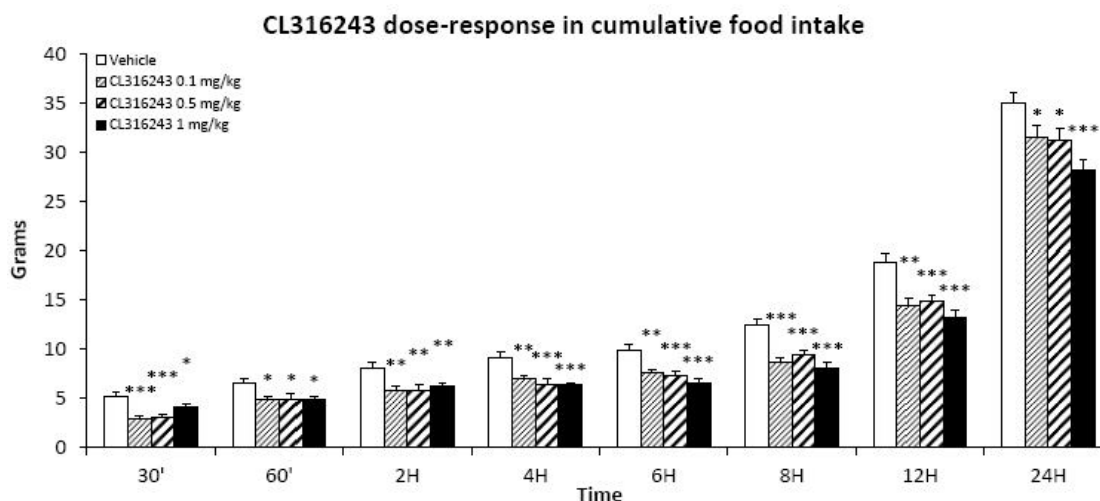


Figure 17. Dose-dependent reduction of food intake after acute administration of the $\beta 3$ -adrenergic receptor agonist CL31543 in Wistar rats.

When combined with either OEA or liraglutide, this $\beta 3$ -adrenergic receptor agonist is capable of reducing the fat mass. Analysis of the thermogenic response using an infrared camera revealed that net temperature of the adipose tissue rose more than 1.5 °C as result of the combined therapy. The analysis of the effects indicated that the combinatorial therapy altered the respiratory quotient towards the oxidation of fat, increased energy expenditure, increased average body temperature, and resulted in the mobilization of resources towards the adipose tissue. Analysis of both, white and brown adipose tissue revealed the reduction of fat content, the increase in the expression of uncoupling protein 1 and the induction of metaplasia white to brown in the abdominal adipose tissue. These results clearly point to an enhancement of thermogenesis and a blockade of the adipogenesis/fat depot as depicted in the following figures.

Overall, these findings indicate that there is a possible utility of combinational therapies based on $\beta 3$ -adrenergic receptor agonists at doses that may not be producing cardiovascular effects. The mechanism of action of this therapy is based on the induction of metaplasia of the white adipose tissue that is transformed in brown-like adipose tissue.

The evaluation of the combinatorial therapy in animal models suggested the induction of thermogenesis and metaplasia brown to white for explaining the weight loss and the reduction of total body fat after a chronic treatment with the combination of OEA and the $\beta 3$ -adrenergic receptor agonist CL31543. In order to clarify the mechanisms by which these metabolic changes occur, we addressed the analysis of the main changes in the oxidative machinery of the adipocyte, the induction of mitochondriogenesis and the appearance of factors promoting the trans-differentiation from a white phenotype to a brown phenotype.

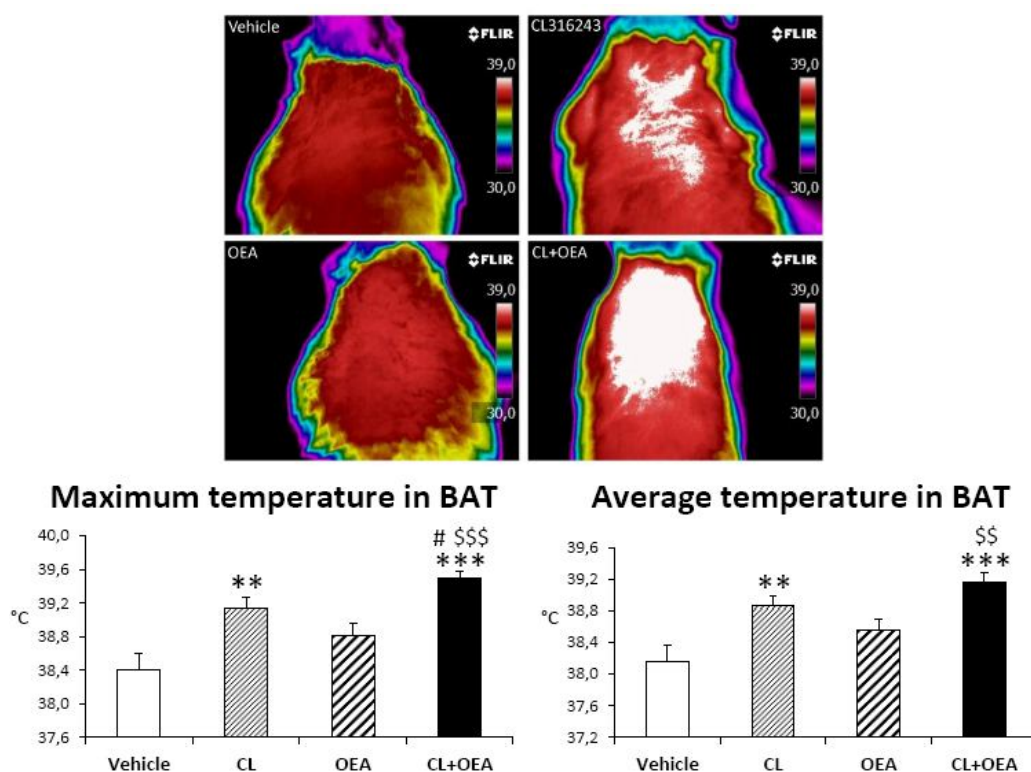


Figure 18. Increase in thermogenesis in dorsal brown adipose tissue after subchronic administration of the β_3 -adrenergic receptor agonist CL31543 combined with OEA in Wistar rats.

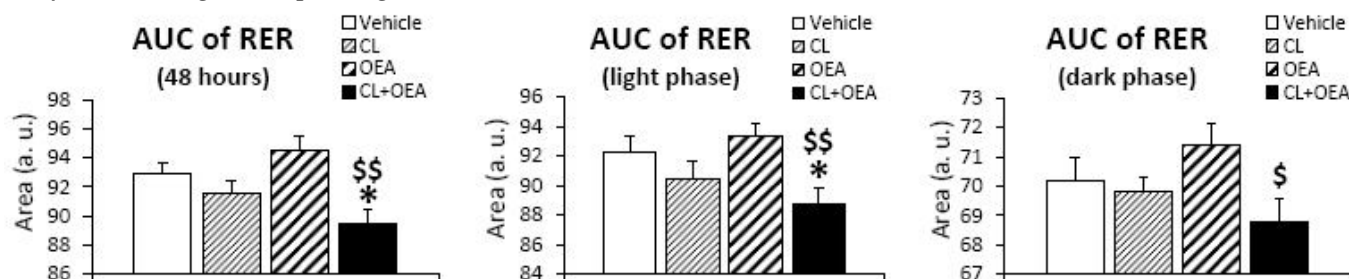


Figure 19. Combinational therapy based on CL31543 + OEA resulted in alteration of the respiratory quotient, indicating greater oxygen consumption.

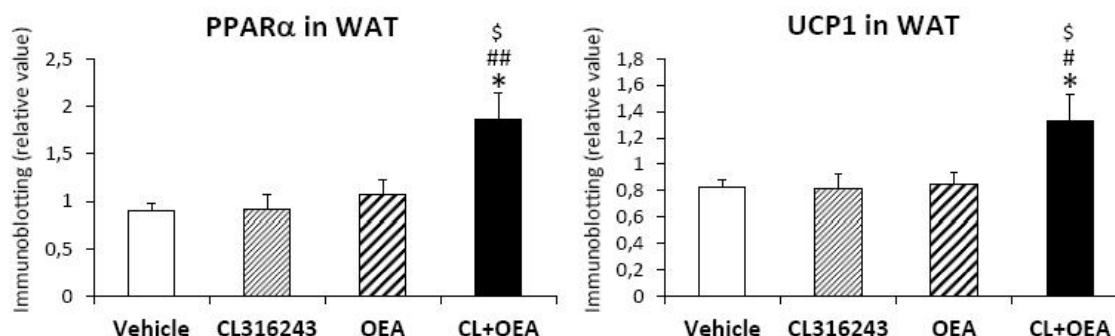


Figure 20. Combinational therapy based on CL31543 + OEA resulted in the induction of white to brown adipose tissue as revealed by the induction of UCP1 protein in white adipose tissue. The induction of PPAR α protein suggests the promotion of oxidative phenotype.

The results indicate that the combinatorial therapy induced an oxidative phenotype thanks to the activation of the PPAR α receptor that results in the induction of the oxidative enzyme ACOX and the transporter CPT1 that promotes the mitochondrial oxidation of fatty acids. This increased oxidative phenotype is associated to an increase in the Srebf1-regulated lipogenic genes FASN and Hmgcr. However, the increased lipogenic activity seems to be directed towards oxidation in the mitochondria, since there is a net reduction of circulating triglycerides and non-sterified fatty acids. This increased utilization of fat in the adipose tissue is not complemented by glucose uptake, since the treatment produces a clear increase in both, blood glucose and circulating insulin, indicative of insulin resistance. These changes are associated with an increase in the number of mitochondria in the adipose tissue, supporting also the white to brown metaplasia observed in the histological analysis. This is confirmed by the increased expression of cytochrome oxidase 4. Additionally, the expression of UCP1 is boosted, indicating that the oxidation of fatty acids in the BAT and WHAT is oriented to heat production, a function typical of brown adipose tissue. This metaplasia is confirmed not only histologically and functionally, but also at the level of the regulatory proteins promoting trans-differentiation Fgf21 and Prdm16.

3. Non patentable discoveries that may be useful to improve clinical management of obesity

A relevant part of the work performed led to the generation of a very useful knowledge that being non patentable, can help the medical community to improve their knowledge on obesity and improve the management of obese people. Two examples of these non-patentable research are a) The in depth analysis of the utility of monitoring plasma or salivary endocannabinoids, b) the explanation of the role of known targets in appetite control, such is the case of central cannabinoid receptors, or c) the evaluation of the potential utility of natural product / nutraceuticals in obesity, such is the case of soy isoflavones.

Monitoring plasma or salivary endocannabinoids

Several evidences have been produced in the last years about the associations between circulating endocannabinoids and pathologic conditions related to metabolic as well as neurologic and immune dysfunctions. However, the definition of the normal endocannabinoid levels, supposed to reflect the endocannabinoid system physiologic activity, still has not been clarified. Besides, alarming incoherence exists among values reported so far for control subjects, to which both analytical, pre-analytical variability, as well as subject inclusion/exclusion criteria, may contribute. As an example, the procedure, timing and temperature of preservation of blood withdrawn can affect dramatically endocannabinoid levels.

A two-dimension liquid chromatography – tandem mass spectrometry (2D-LC-MS/MS) method was developed for the simultaneous measurement of plasma endocannabinoids and related compounds such as arachidonoyl-ethanolamide (AEA), palmitoyl-ethanolamide (PEA) and oleoyl-ethanolamide (OEA), belonging to the N-acyl-ethanolamide (NAE) family, and 2-arachidonoyl-glycerol (2AG) and its inactive isomer 1-arachidonoyl-glycerol (1AG) from the monoacyl-glycerol (MAG) family. The method was fully validated according to Food and Drug Administration guidelines. The on-line purification followed by exhaustive chromatography provided optimal performance and robustness for routine use. Sample collection, storage and processing were carefully assessed. We observed that both NAE and MAG levels dramatically increase in whole blood and in plasma, respectively, thus representing a major source of variability. Hence, the immediate centrifugation of blood and storage of plasma was established for a correct sampling. Moreover, assisted thawing and rapid plasma processing were established for accurate

detection. After testing several extraction procedures, we finally chose a liquid-liquid extraction based on toluene. This solvent yielded optimal recovery preserving the stability of the two isomers 2AG and 1AG. Aiming at establishing gender specific reference intervals of general valence, we recruited 121 healthy subjects fulfilling rigorous criteria: they were all drug-free, normal weight subjects without visceral adiposity; all subjects showed normal diastolic and systolic blood pressure, normal glycaemia and no alteration in the circulating lipid profile. Subjects reported to have a normal wake-sleep cycle, and they successfully fulfilled psychometric tests for depression and feeding behaviour and attitude. Reference lower and upper limits were estimated as the 2.5th and the 97.5th centiles. Males and females showed similar NAE values, but males had higher levels of MAGs. In females, but not in males, AEA level seemed to be associated to adiposity, even at normal weight BMI. Interestingly, a strong correlation was found between MAGs and circulating triglycerides, suggesting that a strong link between endocannabinoid system and triglycerides exists independently of a condition of obesity or overweight, and might represent an early trigger for the development of lipid metabolism alteration.

Our data indicated that even in a homogeneous cohort of healthy subjects some associations with circulating endocannabinoids exist, and that the kind and the extent of these associations are strictly gender-dependent. By using the gender specific upper reference limit we analysed the prevalence of altered AEA and 2AG values in the general population by measuring plasma from 348 males (M) and 439 females (F) (106 and 160 normal weight (NW), BMI<25.0kg/m²; 242 and 279 overweight/obese subjects, BMI≥25.0kg/m²) aged 18–90y. We found that the prevalence of altered endocannabinoid values is higher in females than in males, and that increased AEA and 2AG levels are more frequent in overweight/obese subjects than in normal weight, (M: 9.5 vs 3.8%, F: 29.4 vs 9.4% for AEA; M: 8.7 vs 3.8% and F: 17.2 vs 9.4% for 2AG). In overweight/obese subjects, increased endocannabinoid values seem to be associated with altered metabolic parameters: males with elevated 2AG showed similar BMI but higher diastolic and systolic blood pressure (DBP and SBP, p=0.018 and p=0.006, respectively) and triglycerides (p=0.036) compared to those having 2AG in the normal range. Females with higher 2AG showed increased BMI (p=0.012), glycaemia (p=0.020) and triglycerides (p<0.0001), the latter maintaining the statistical significance after correction for BMI (p=0.0005). Both males and females with elevated AEA exhibited higher BMI and waist circumference compared to those with AEA in the normal range (p=0.025 and p<0.0001; p=0.014 and p=0.001, respectively). Females showing higher AEA had increased DBP, SBP and insulin even after correction for BMI (p=0.006, p=0.016 and p=0.032, respectively).

DEFINITION OF THE NORMAL ENDOCANNABINOID TONE			
ESTIMATION OF EC REFERENCE INTERVALS			
		Median (Iq)	Reference Interval 2.5 - 97.5 P
AEA (pmol/ml)	F	0.99 (0.79 - 1.18)	0.57 - 1.59
	M	0.98 (0.88 - 1.10)	0.54 - 1.66
PEA (pmol/ml)	F	14.35 (12.70 - 18.00)	9.93 - 23.88
	M	15.20 (13.3 - 17.05)	8.70 - 21.00
OEA (pmol/ml)	F	4.74 (4.03 - 5.91)	2.72 - 8.07
	M	4.54 (3.66 - 5.86)	2.58 - 8.61
2AG (pmol/ml)	F	1.43 (1.06 - 1.90)	0.71 - 3.12
	M	1.73 (1.37 - 2.39)**	0.73 - 4.23
1AG (pmol/ml)	F	0.49 (0.38 - 0.64)	0.25 - 0.95
	M	0.58 (0.43 - 0.76)	0.28 - 1.43

*: P < 0.050; **: P < 0.010; ***: P < 0.001.

Fanelli et al., 2012

Figure 21. Reference values for plasma endocannabinoids in the Massa-Lombarda Study.

In conclusion, associations between circulating endocannabinoids and adiposity and metabolic parameters exist even in healthy normal weight subjects, suggesting that they might be involved in the early stages of the dysmetabolism development. This finding is strongly supported by the observation that in overweight/obese subjects the alterations of circulating endocannabinoids are associated to cardiometabolic risk factors independently from adiposity. Since different prevalence in endocannabinoid alteration and different metabolic dysfunction were observed in males and females, gender specific mechanism should be postulated.

Endocannabinoids in saliva

Plasma endocannabinoid levels can be considered as biomarkers of obesity (see previous work). However, the collection of plasma samples is laborious and need accurate control conditions. Therefore, we evaluated the possibility that endocannabinoids could be measured in more accessible samples such as saliva. The results reveal that plasma endocannabinoids might even be considered as better biomarkers of obesity than plasma levels, due to their more stable levels in relationship to nutritional state.

To this end we explore endocannabinoids and related N-acylethanolamines in saliva and verify changes in relation to body weight status and in response to a meal or to body weight loss. We measured fasting plasma and salivary endocannabinoids and N-acylethanolamines through liquid mass spectrometry in 12 normal weight and 12 obese insulin-resistant subjects. Salivary endocannabinoids and N-acylethanolamines were evaluated in the same cohorts before and after the consumption of a meal. Changes in salivary endocannabinoids and N-acylethanolamines after body weight loss were investigated in a second group of 12 obese subjects following a 12-weeks lifestyle intervention program. The levels of mRNAs coding for enzymes regulating the metabolism of endocannabinoids and of cannabinoid type 1 (CB1) receptor, together with endocannabinoids and N-acylethanolamines content, were assessed in human salivary glands. The endocannabinoids 2-arachidonoylglycerol (2-AG), N-arachidonylethanolamide (anandamide, AEA), and the N-acylethanolamines (oleoylethanolamide, OEA and palmitoylethanolamide, PEA) were quantifiable in saliva and their levels were significantly higher in obese than in normal-weight subjects. Fasting salivary AEA and OEA directly correlated with BMI, waist circumference and fasting insulin. Salivary endocannabinoids and N-acylethanolamines did not change in response to a meal. CB1 receptors, ligands and enzymes were expressed in the salivary glands. Finally, a body weight loss of 5.3% obtained after a 12-weeks lifestyle intervention significantly decreased salivary AEA levels. As a conclusion, endocannabinoids and N-acylethanolamines are quantifiable in saliva and their levels correlate with obesity but not with feeding status. Body weight loss significantly decreases salivary AEA independently of feeding status, which might represent a useful biomarker in obesity.

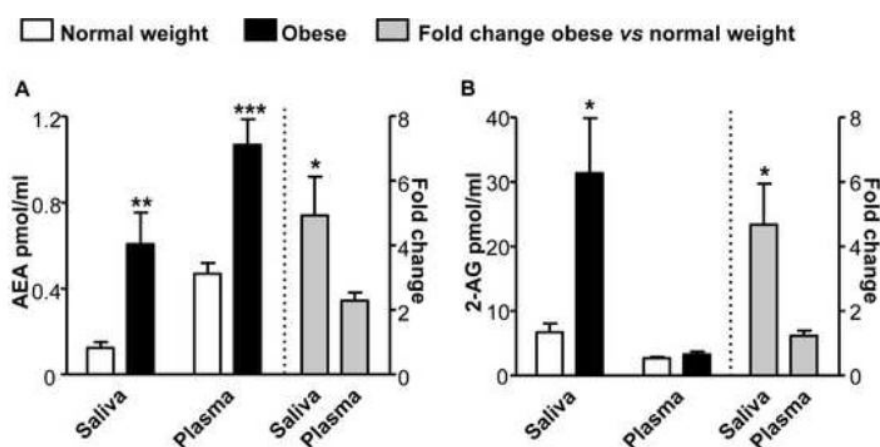


Figure 23 Salivary endocannabinoids in normal and obese subjects.

Central cannabinoid receptors controlling appetite

Activation of CB₁ receptors is universally recognised as a powerful endogenous orexigenic signal. However, pharmacological blockade and full genetic inactivation of CB₁ receptors induce effects on food intake that are transient and not very pronounced, respectively. This could be due to the fact that CB₁ receptors are able to decrease both excitatory and inhibitory neurotransmission in brain regions that regulate food intake. These opposite effects might obviously have opposite impacts onto food intake. We investigated whether cell type-specific CB₁ deletion might clarify the role of this receptor on food intake. To do this, we used the Cre/loxP system to generate CB₁ conditional mice lacking the expression of the receptor either in cortical glutamatergic excitatory neurons (Glu-CB₁-KO mice) or in inhibitory GABAergic neurons (GABA-CB₁-KO). We stimulated food intake in mutant animals by acting on two distinct motivational mechanisms. To enhance the “drive” to eat, we measured food intake during refeeding after a 24-h food deprivation. To stimulate the “incentive” component of food intake, we exposed fed mice to a palatable food. In both conditions, Glu-CB₁-KO mice ate less than controls, whereas surprisingly GABA-CB₁-KO mice ate more than the controls. Our data also showed that the well-known biphasic effects of CB₁ agonists, with low doses exerting hyperphagia and high doses hypophagia are due to specific actions of the different doses on CB₁ expressed on excitatory or inhibitory neurons, respectively. These findings were published in *Nature Neuroscience* (Bellocchio et al., 2010, in press) and in *Neuropharmacology* (Lafenetre et al., 2009), and revealed two unexpected opposite brain functions of CB₁ receptors in the regulation of stimulated food intake. First, the control of glutamatergic transmission by CB₁ receptors is responsible at least in part for the well-known orexigenic role of the endocannabinoid system (ECS). Second, CB₁ receptors expressed on GABAergic neurons mediate, by reducing local inhibitory transmission, a previously unknown inhibitory function of the ECS on stimulated food intake. Moreover, unpublished results indicate that the anorectic effects of the CB₁ antagonist Rimonabant are not mediated by either the two populations, but are abolished in mice lacking CB₁ expression in forebrain principal neurons, suggesting that the feeding-related effects of agonists and antagonists of CB₁ are mediated by different neuronal populations (e.g. serotonergic, noradrenergic, dopaminergic, or cholinergic neurons). These results suggest also that drugs targeting CB₁ expressed in specific neuronal populations will represent anti-obesity agents more efficient and probably devoid of side effects. These results also indicate that such drugs can actually be developed since different populations of receptors respond differently to pharmacological agents. Moreover, this concept could be possibly extended also to peripheral CB₁ receptors.

Utility of natural products: the case of soy isoflavones

The lack of safe and effective approved drugs against obesity has raised a great interest on natural products that may serve as alternative therapies. From this perspective we have addressed the analysis of several products, including the polyphenols hydroxytyrosol, hydroxytyrosol acetate and resveratrol as well as the effects of daidzein, one of the main soy isoflavones, on diet induced obesity in rats. Data on polyphenols only indicated an improvement on circulating cholesterol levels. However data on daidzein were more surprising, and support its use as a healthy nutritional supplement to diet. Rats made obese after long term exposure to a very (60%) high fat content diet were treated with Daidzein (50 mg/kg) for 14 days. The dose was selected on the basis of the acute effects of this isoflavone on a feeding test. After 14 days, animals were sacrificed and plasma, adipose tissue, muscle and liver studied for the levels and expression of metabolites and genes relevant for lipid metabolism. Acute daidzein dose-dependently reduced food intake. Chronic treatment reduced weight gain and reduced fat content in the liver. This was associated with high leptin levels and low adiponectin contents in plasma. While skeletal muscle was weakly affected by treatment, both adipose tissue and liver displayed marked changes induced by daidzein that affect transcription factors and lipogenic enzymes, specially stearyl coenzyme A desaturase-1, a pivotal enzyme in obesity. Additionally, Daidzein induced thermogenesis by promoting the expression of uncoupling protein 1. These results give support to the use of isoflavones in diet induced obesity, especially when hepatic steatosis is present, opening a new field of use for these natural products.

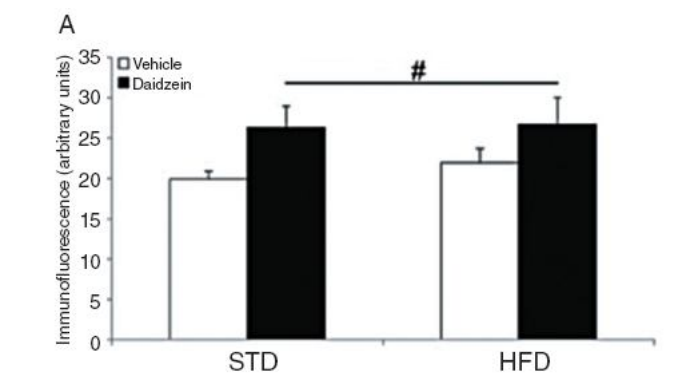


Figure 24. Chronic treatment with the isoflavone Daidzein promoted the expression of UCP-1 in brown adipose tissue after exposure to normal or high (HFD) diet. Data is specific immunofluorescence against UCP-1

4. Scientific reports and reports oriented to the community

Reprobesity has been an extremely productive consortium. More than 50 peer review papers have been published in addition to the 9 industrial patents. Multiple dissemination actions from academic meetings to mass media reports have been addressed, covering all the aspects of the field of obesity, from clinical to basic research. All the dissemination actions can be examined in the open access web page www.reprobesity.eu.

1.4 Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results.

Socio-Economic impact and Societal implications

Reprobesity was a project focused in a SME, with the main objective of generating exploitable foreground, mainly as two classes of products: a) Candidates to enter into clinical trials for obesity therapeutics and b) Technology for identifying biomarkers suitable for a better stratification of patients suffering complicated obesity. Both objectives have been achieved, and the SME VIVIA is taking the challenge of starting Phase 1 clinical trials with the 1,2,4-ozadiazol allosteric modulator VIVIA011.

The identification of these active compounds and that of its enantiomers identified a new class of therapy based on the allosteric modulation of GLP1 receptors. All the active compounds tested, which are GLP-1 receptor potentiating compounds could be therapeutically beneficial in monotherapy or in combination therapies. Data obtained along project development indicate a clear activity when administered alone as monotherapy and at low doses. In addition, these compounds could also be therapeutically beneficial in combination with other treatments. There are two preferential classes of diabetes drugs to consider for combination therapies with these allosteric potentiator compounds. The approved GLP-1-like peptidic drugs exendin-4 (Exenatide) and Liraglutide that act as direct agonists to the GLP-1 receptor, and inhibitors of DPP IV protease that cleaves the endogenous GLP-1 peptide.

The allosteric potentiating compounds can actually potentiate also GLP-1-related peptidic drugs like exendin-4 that are commercialized drugs for diabetes. It has been reported in the literature that the same compound may not potentiate different agonists in the same receptor and in particular in GLP-1 receptor (Leach et al; Trends Pharmacol Sci 2007; 28: 382-389; Langmead and Christopozlos; Trends Pharmacol Sci 2006; 27:475-481 and Kenakin; Trends Pharmacol Sci 2009; 30:460-469). Thus, it cannot be assumed that a compound that potentiates GLP-1 would in any obvious deduction also potentiate exendin-4 or e.g. liraglutide. Thus, the present inventive compounds not only potentiate endogenous GLP-1 peptide but also exogenously added GLP-1 receptor peptidic agonist drugs. For diabetes, a combination therapy of the present inventive compounds with exendin-4, potentiating the effects of exendin, would offer higher efficacy at lower doses, which could benefit the patients with higher safety for long term treatment.

An additional unexpected therapeutic benefit results as the present inventive allosteric compounds potentiate the effect of exendin-4 to the point where they become efficacious as anti-obesity drugs. Major efforts have been under way to show efficacy of these peptidic drugs in weight loss fight against a necessary significant increase in dosing, which may compromise their safety profile for long term treatment. The present inventive compounds potentiate these peptidic drugs to be efficacious in weight loss at tolerable doses, which means an important benefit for obese patients. In particular, there is a large co-morbidity between diabetes and obesity and thus such a combination treatment will have a major impact in patients' health and wellbeing. Obesity efficacy has been tested in animal models using an initial fasting model: 24 hour food-deprived rats. Thus, an intraperitoneal administration of compound VIVIA011 combined with a sub-effective dose of exendin-4 reduces food intake in food deprived Wistar.

The therapeutic benefit of a combination of the present allosteric potentiators of the GLP-1 receptor and DPP IV inhibitors can be predicted on the basis of their respective mechanisms of action. DPP IV inhibitors inhibit the protease that cleaves endogenous GLP-1, DPP IV, resulting in higher levels of endogenous GLP-1 peptide. This additional GLP-1 peptide would be expected to be equally potentiated

by the present GLP-1 allosteric potentiators. Such potentiation will enable a high efficacy combination of small molecule oral drugs, cost-effective with future generic DPP IV, with potential benefits both in diabetes and obesity. As described above, the main significant result for REPROBESITY is the identification of 1,2,4-oxadiazole derivatives as safe and effective therapies against Diabetes Type 2 associated to obesity. VIVIA Biotech is undertaking the aim of launching a Phase 1 clinical trial along the first semester of 2013.

The societal implications of these achievements are very relevant, since in the last 4 years almost all drugs entering into phase III of clinical trials have to be removed from further development because of safety issues. The 1,2,4 oxadiazols, being allosteric potentiators, display no intrinsic activity but a positive modulation of an endogenous signal, allowing a much safer profile than previous drugs under development. Whether these new class of drugs will be as effective as expected from its conception, needs a clinical trial confirmation. But, nonetheless, to reach clinical trials in 42 months of research is a landmark in preclinical development and set in place the real value of the multidisciplinary academic-SME collaboration at the level of the European Union.

Main Dissemination activities

Reprobesity Consortium has been extremely careful on communicating main findings of the project to the community, disseminating relevant information after protecting/patenting the data. This has been accomplished by means of performing the following tasks:

- 1 Internal /external communication: A web page will be created for rapid dissemination of knowledge to the community. It will have a secure site for internal distribution of information.
- 2 A strategy of publication of scientific reports and community-oriented reports will be established by the consortium.
- 3 A strategy of public events (conferences, workshops, etc...) will be adopted to facilitate dissemination of knowledge.

Dissemination activities was led by Coordinator Institution, IMABIS, that has aimed at increasing project impact through a set of dissemination activities while ensuring a strong engagement of various stakeholders in the project. It has included more precisely the following tasks:

a) The Project Website

The project website (www.reprobesity.eu) is being the key tool to ensure the largest project impact and dissemination. Among its main characteristics: easy to use, content oriented, including collaborative features (Web 2.0). All along the project period, any public project content have been made available to visitors as soon as they have been produced, allowing to keep visitors fully informed of the life of the project (including project activities and results, and contribution to events).

The overall responsibility of designing, updating, and operating the website will be the one of the Workpackage leader, specifically it was carried out by the project manager, but all partners have been asked to validate the website specification and to contribute to its content development.

A comprehensive set of the project information has been created by means of different actions (press releases, flyers, subcontracting specialized report...). All the information is available online in the different sections of the Website.

b) To Publish results in Scientific Journals and Books

Each partner has, within this task and over the project lifetime, contributed to the publication of articles, based on project rationale, methodology and outputs.

c) To Communicate News about the Project and Obesity

In order to improve this task, during this period, the website was remodeled and the new section “Communication” (top menu) was created. In this section the visitors can find the submenus:

- Project News, here the news, press releases, project meetings are published.
- Reprobesity in media, with Websites linking to REPROBESITY.
- Obesity News, to inform visitors and REPROBESITY consortium about new obesity research results



Figure 25. Snapshot of the section “Project News” of the REPROBESITY Website

d) To Communicate the Results in Scientific Congresses

Accepted or foreseen contributions to congresses, workshops and conferences were reported on a trimestral basis. It is also within this task that the project coordinator has encouraged the participation of the project in events organised by European bodies, scientific associations or federations.

e) To Present the Project in Workshops

Another way for a project to have an important impact and in consequence to be able to efficiently spread excellence and disseminate knowhow has been the participation in specialized events (industry or policy makers) to present activities, the consortium and the development of the project itself and the impact of results to the business sector.

f) To Elaborate public community-oriented reports

This task is detailed in deliverable 22. REPROBESITY has additionally carried out dissemination into the wider public through the web page, flyers, interviews, articles in popular press, targeting regional and European public. The consortium produced appropriate project results material for presentation

opportunities that arise, addressing them to targeted stakeholders groups, and tailoring the language and levels of complexity for the intended audience.

g) To improve internal communication

The aim of the internal communication has been to promote an increasing enthusiasm for receiving the results when they become available. For this purpose, the REPROBESITY Website was designed divided into a public area and a password protected partner area (intranet) in which all relevant and confidential documents are provided to all partners.

As a result of the dissemination tasks, the following results have been obtained:

REPROBESITY Website

During this period there has been an increase in Internet activity, with an improvement in all the analyzed data.

Table 1. Comparison of Internet activity data from Period 1 and Period 2.

Activity data	June 2009-May 2010	June 2010-May 2012
Total visits	2390	6339
Average visits/month	200	270
Google Results	84	317
Visiting countries	65	90

Table 2. Number of visits from different countries.

Spain	3501
United States	620
Italy	357
United Kingdom	296
Germany	261
France	198
India	106
Belgium	77
Canada	75
Netherlands	58

Publications on scientific journals or books

Over the duration of the project, each partner except the company (Vivia Biotech) has successfully submit and published articles in scientific journals or books, some of them of very high impact factor as Nature Medicine. A total of 40 articles have been published in this period 10 of which were published in Open Access Journals. In the same way, 3 doctoral theses have been presented as a training result of the project:

04/07/2011. Caracterización de las acciones antiobesidad de los antagonistas cannabinoides AM251 Y LH21. Ana Crespillo. Universidad de Málaga. (Spain)

09/09/2011. El sistema endocannabinoide en el hipocampo de rata y su modulación por factores epigenéticos. Patricia Rivera. Universidad de Málaga. (Spain)

19/12/2011. The cannabinoid receptor type 1 and the body's energy homeostasis; Ph.D. thesis submitted to the Johannes Gutenberg. (Germany)

Communication of News about the Project and Obesity

A total of 20 project news were published on the Website, all the consortium meetings were announced and partners published some job offers. In the same way, a total of 21 news on obesity research results over the world were published. Finally, some links to communication media which referenced to reprobesity were published on section REPROBESITY in media.

Communication of Results in Scientific Congresses

Was carried out active work in the presentation of results at conferences and national and international scientific meetings, with exposure of 12 lectures and 12 posters presenting research results.

Presentations of REPROBESITY

The Project has been presented in two Spanish national workshops. All the **documentation presented in workshops** is accessible to the community on the section “Results/Dissemination” of the REPROBESITY website. During this period, the project has been presented in the following events:

- 21/10/2011. Cádiz (Spain). 9 Jornadas Salud Investiga Fundación Progreso y Salud. Fernando Rodríguez de Fonseca.
http://www.jornadasaludinvestiga.es/resources/archivos/jueves/FERNANO_RODRIGUEZ_DE_FONSECA.pdf 16 DOWNLOADS
- 15/07/2010. Córdoba (Spain). Taller Práctico de Preparación de Propuestas al VII PM: Sector Salud. Citandalucia. Miguel Romero Cuevas. <http://www.citandalucia.es/sites/default/files/5-Proyecto%20REPROBESITY%20Miguel%20Romero.pdf.pdf> 12 DOWNLOADS

Development of REPROBESITY materials

The following materials have been produced for display at conferences and online:

- Powerpoint presentations – giving information about REPROBESITY activities and results (i.e. flyers Figure 2).
- Posters – Some posters have been produced giving information about REPROBESITY and also announcements of meetings and other events
- A key dissemination action has been the especial article published on the specialized journal International Innovation of the portal researchmedia.eu web portal. The whole consortium participated in this action, a the article “The obesity remedy” was produced and distributed to all stakeholders among the European Union



Figure 26. Flyer of REPROBESITY description and main results.

Internal Communication

Finally, the intranet of the website was launched as tool for internal communication, where all the researchers of the consortium can download all internal and confidential documents. However, the main internal communication tool has been the e-mail.

1.5 Public website address (if applicable), as well as relevant contact details.

The web page of the consortium is: www.reprobesity.eu