TITLE:

New energy-sensing metabolites: Beneficial effects on metabolic health in obesity comparing diary caloric restriction vs intermittent fasting. A randomized cross-over study

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HEALTH RESEARCH PROJECTS

Application No
PI20/00338

TITLE: **New energy-sensing metabolites: Beneficial effects on metabolic health in obesity comparing diary caloric restriction vs intermittent fasting. A randomized cross-over study**

ABSTRACT (Objectives and Methodology of the Project) **(Please only use the space provided below)**

General integrated goal of the coordinated project:
To elucidate the role of succinate and other metabolites derived from the intestinal microbiota such as Short Chain Fatty Acids (SCFAs), as energy sensing metabolites in the context of obesity and type 2 diabetes (T2D).
Specific objectives of Subproject 1 (SP1): **1a**. - To investigate whether intermittent fasting (IF) is better than Continued Daily Caloric Restriction (DCR) in terms of metabolic improvement through the study of: 1) the dynamics of gastrointestinal hormones and energy sensing metabolites, 2) the intestinal microbiome, 3) variability on succinate and SCFAs, MCFAs and Biliary Acid after weight loss; **1b.** - To characterize the functional changes of adipose tissue mesenchymal derived stem cells (ASCs) in IF and DCR conditions according to the BMI of the donor (ex vivo study); **1c.**- To investigate the expression and secretory profile of ASCs according calorie restriction conditions.
Methodology: clinical study: randomized, cross-over design, study participants (n=15) will consume either lifestyle recommendations for a healthy Mediterranean diet under a continued caloric restriction diet (**DCR**) or will undertake an intermittent (**IF)** protocol. Clinical, anthropometrical and functional studies. Metabolomics for gut derived metabolites in plasma. Enteroendocrine gastrointestinal dynamics. Metagenomic analysis.

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HEALTH RESEARCH PROJECT APPLICATION FORM
BACKGROUND AND STATE-OF-THE-ART

Project's aim, background and state-of-the-art of the scientific and technical knowledge, and national or international groups working in the same specific or related lines.
Please state references in the next item: Relevant references **Max. 3 pages (15,700 characters)**

Obesity and type 2 diabetes (T2D) are directly responsible for a high percentage of mortality among "non-communicable diseases" (https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases), with more than 2 million deaths annually worldwide. Their prevalence, despite all efforts to prevent and treat them, continue to **increase** relentlessly, becoming one of the main **health and social emergencies** for the healthcaresystems. There are many causes of this **epidemic,** and better understanding the reasons that lead to them is critical to better and more efficiently prevent and treat these diseases. Weight control is a crucial element involved in both diseases (obesity and T2D) and despite being the subject of study for decades, **its management** remains **elusive** in most interventions.

Many elements are involved in weight control, ranging from (a) alterations **of the energy balance** with high **inter-individual variability** [1,2] in terms of metabolic flexibility defining "***thrifty***" and "***non-thrifty***" phenotypes [3], (b) hormone mediators such as **FGF21** identified in animal models and suggested in associative studies of obese patients [4], (c) changes in **adaptive thermogenesis** although the **impact of brown tissue** in humans in energy expenditure is limited**,** (d) **genetic factors** with an effect on the overall result of energy expenditure, and (e) **energy sensors** responsible for establishing a direct link between energy expenditure and intake [5-8]. In this latter area, **new elements** are emerging as relevant players with a high potential to become useful tools both to better **understand the pathophysiological** process and to delineate useful **therapeutic approaches.**

Energy sensing metabolites:

There is increasing evidence that there is a direct relationship between energy expenditure and intake, as a key element in determining daily energy balance [7,8], through what is known as energy-sensing mechanisms [7]. These mechanisms, which are still little known, may include **biological mediators** such as certain **hormones** like myokines, adipokines and **metabolites** such as **glucose** itself, **bile acids** or **free fatty acids** with a central action on intake regulatory centers [9]. The **maladjustment** between **nutrient availability and cell energy requirements** is a key factor contributing to the development of **obesity** and **T2D**. **Dynamic exchanges of intra- and extracellular metabolites are crucial** to duly integrate and **coordinate** the biological network in cells, particularly **the concentration of nutrients andintermediate metabolites** [10].

Succinate may have a new role in energy sensing:

There is wide evidence that succinate is a **pleiotropic metabolite** that works not only as an energy intermediate but also as a signalling metabolite both in cytosol, but also and extracellularly through its cognate receptor **SUCNR1** [11]. Although it has been long been described as a danger signal, recently, in the context of energy homeostasis, succinate has shown to be an antilipolytic factor [12], a potent activator of thermogenesis [13] and a regulator of intestinal gluconeogenesis [14, 15]. Recently, we have demonstrated that **succinate** acts as a new **signaling metabolite** that **controls** the **resolution of inflammation**, a physiological **circuit that is broken in obesity**. Thus, we have observed that SUCNR1 deficiency in macrophages induces *in vivo* inflammation, glucose intolerance and cell metabolic stress [16]. **High levels of circulating succinate** have been mainly described in pathological conditions [17-20], including **obesity and T2D** [17,18]. Therefore, it is not unreasonable to suggest that obesity may be associated with a **resistance to succinate** as occurs with other hormones such as insulin or leptin [21,22], favoring a vicious circle of higher succinate levels, greater succinate resistance, which has already been proven in terms of its effects on the resolution of

inflammation [16]. However, circulating succinate does not only increase in diseases and data from previous studies also **describe higher levels of succinate** in some physiological conditions, such as **enduring exercise** [23].
Therefore, despite all the available information, the physiological role of succinate in the energy balance and in the pathophysiology of obesity and related diseases needs to be clarified. In fact, the **main source of succinate circulating levels** is so far **unknown**, but there is strong evidence pointing to the **intestine** as one of the **relevant contributors** to blood levels, through differences in the composition of the microbiota [18].

Circulating succinate as a biomarker of metabolic health:

This metabolite is an excellent marker of cell state as it occupies a key position in the metabolism, being the only mediator between the tri-carboxylic acid cycle and the mitochondrial respiration chain through complex I. **Succinate** is a good marker of cell damage and circulating levels have been used as predictors of **poor prognosis** in critically ill patients, and as a marker of **ischemia and inflammation.** When mitochondrial dysfunction occurs, as has been proved in metabolically active tissues in obesity and T2D, mitochondrial succinate levels increase, and subsequently extracellular levels could also increase [24, 25]. Thus, in a pathological context **circulating succinate** might be **sourced from both damaged tissues and gut microbiota.**
Our results [26] point to **succinate** as a metabolite capable of **representing** at the same time various components of the metabolic state, offering a broader vision of **energy homeostasis**, contained in a single, easily measurable **marker**.

Succinate, a metabolite regulated by dietary intake:

Metabolomic analysis of plasma after a meal shows an increase in succinate, pyruvate, and lactate, indicating a postprandial shift in the body's energy source from β-oxidation to glycolysis [27]. Interestingly and similarly to what happens with some gastrointestinal hormones, we have seen that **this metabolite** presents a **response after food intake** and that this response is **dependent** on both the type of **nutrient** and the **route of administration**, indicating that it has a clear nutritional regulation. Thus, we have observed that there is a **peak** in circulating levels 60' **after a standard meal test**, which is **reproduced** after administering **glucose orally**, unlike what happens when it is administered intravenously, (*preliminary data submitted in Diabetes Care 2020, annex figure 1*). This indicates that the higher levels of circulating succinate after food ingestion are not only consequence of glycolysis and intestine play a key role.

The physiological significance of this dynamic of succinate related to intake requires further investigation. In this regard, the **new concept** that energy sensing metabolites act as **signaling molecules** with **extracellular functions** beyond energy obtention is gaining prominence. In fact, it has been postulated for some molecules such as **short- chain fatty acids (*SCFAs*)** from the intestinal **microbiome**, which can regulate the energy metabolism in the host by exerting an autocrine-paracrine signal on adipose tissue with an **anti-lipolytic effect** [28,29] mediated by the attenuation of the effect of *hormone-sensitive lipase (HSL),* besides other metabolic effects.

Effect of weight loss on succinate levels:

In addition to intake and dysmetabolic situations, **circulating levels** are also likely to undergo **changes** after weight loss. Severely obese patients, with and without dysglycemia, are able to reverse high circulating levels **after weight loss** [26]. In these patients, decreased circulating levels are paralleled with a restoration of the **"physiological"** response after food ingestion observed in non-obese subjects (*Submitted in Diabetes Care, figure 2*). Interestingly, this behavior is **highly like** what we observe with **incretin hormones** after a meal ingestion. This supports the conceptual hypothesis that succinate is a good marker of metabolic health, which can be especially useful as a **biomarker** in certain circumstances as we have already noted in the case of **diabetes remission** following metabolic surgery [26].

In summary, among the mechanisms involved in weight control, energy sensing metabolites are opening new paths to help better understand the concept of metabolic flexibility and propose new therapeutic approaches in diseases such as obesity and diabetes. In this field, typically intracellular energy metabolites such as succinate are now being considered relevant in their extracellular signalling function through their specific receptor in metabolically sensitive

tissues. To date we have sufficient preliminary evidence supporting this function, with data that clearly point to a nutritional response and a more-than-likely intervention of the intestinal flora in plasma regulation.

Specific proposal subproject 1:

Weight loss

Daily calorie restriction diets (DCR)and more recently, diets that incorporate **intermittent fasting (IF),** are suitable methods that can reduce obesity and its associated comorbidities including T2D [30]. Both (DCR and IF), have shown **similar efficacy** in improving body composition, reducing cardiovascular risk factors, improving glycemic control, insulin sensitivity and insulin secretion. The **benefits** of **hypocaloric diets** are dependent on **weight loss capacity and** on the degree of **fat mass reduction**. However, diets involving **IF**, particularly those adapted to the individual's **circadian rhythm** (**eTRF** *early-time-restricted-feeding*), can achieve similar **benefits** even **without weight loss** [31,32].

Metabolic switch

During **fasting periods**, **triglycerides are broken** down to **fatty acids** that are **converted** by the **liver and gut** epithelial cells in **ketone bodies** during both b-oxidation to acetyl -coenzyme A (CoA) and/or conversion of ketogenic amino acids, and are **released into the bloodstream** to provide **metabolic fuel** to various organs [33]. Ketone bodies begin their increase in plasma within 8 to 12 hours after the onset of fasting, reaching levels as high as 2 to 5 mM by 24 hours [34]. **Ketone bodies** are not just fuel used during periods of fasting but also **act as signaling molecules** with relevant cellular and organ effects. Ketone bodies **regulate** the **expression and activity of many proteins and molecules**. These include peroxisome proliferator -activated receptor γ coactivator 1α (PGC-1α), nicotinamide adenine dinucleotide (NAD+), fibroblast growth factor-21, sirtuins, poly-adenosine diphosphate [ADP] -ribose) polymerase 1 (PARP1) and ADP ribosyl cyclase (CD38) [35-39], among others. All of these changes drive the switch on systemic metabolism.

Health benefits of intermittent fasting

As commented above, many studies have indicated that several of the **benefits** of **IF** are **dissociated** from its effects on **weight loss**. In the recent work by Sutton EF et al. in pre-diabetic patients, all classic metabolic parameters except triglyceride levels improved during intermittent fasting, without weight loss [40]**.** Most organ systems respond to **IF** to enable the organism to overcome the challenge and **restore homeostasis**. Intermittent fasting **induces** an **adaptive stress response** leading to an increase in cellular DNA repair mechanisms, protein quality control, antioxidant defenses, mitochondrial biogenesis and autophagy, and inflammation [41]. Although we do not fully understand the precise mechanisms, the **beneficial effects of IF** have been attributed to a **metabolic switching** and an improvement in the cellular response to a persisting stress**.** Notably**,** although it is widely thought that changes in the microbiome might explain some of the beneficial effects of IF**,** nowadays **no in-depth analysis** have been done **to address** the effects on **gastrointestinal hormones, energy sensors** and the **microbiome**. Studies in **animal models** demonstrate an **improvement** in **systemic inflammatory parameters and in adipose tissue with improved thermogenic** [42] **response** and the **restoration** of the dynamics in **intestinal flora** improving metabolic [43] health, when subjected to IF. We know today that **commensal bacteria** and **their metabolites** have the potential to exert **pro- or anti-inflammatory effects** regulating the immune response in the **intestinal wall** [44]. Finally, these changes may have **systemic effects**, as already described in some autoimmune and neurological [45] diseases. Continued low-calorie dieting has clear systemic anti-inflammatory effects [46], although changes in the microbiome are less clear. In **our series** of patients put on **continued low-calorie dieting for 6 months** with effective weight loss, an **improvement** is noted in all**metabolic parameters** but with great **interindividual variability in the microbiome** between before and after weight loss (*preliminary data attached, figure 3*). Changes during IF have only been explored in **animal models** with an **increase** in the relative abundance of **bacteria associated with the synthesis and degradation of ketone bodies** and metabolic pathways involved in the **glutathione metabolism** [47], which could favour **antioxidant pathways** that would eventually influence the host.

We think that the **recovery of intestinal microbiome** following an **IF diet in obese patients,** could favor an **anti-inflammatory metabolic profile in the microbiome** that will eventually be **transmitted to the host**. The **recovery** in the **response** of the **gastrointestinal hormones** and in **metabolic sensors** such as **succinate or SCFAs** could be behind the differences between the two types of calorie restriction.

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HEALTH RESEARCH PROJECT APPLICATION FORM
BACKGROUND AND STATE-OF-THE-ART. RELEVANT REFERENCES

Please list references of the quotes included in the previous item: background and state-of-the-art. (Max. 1 page)

[1]. Schlogl M, et al. Diabetes 2015;64:3680-3689
[2]. Rynders CA, et al. Obesity (Silver Spring) 2017;25:873-880
[3]. Reinhardt M, et al. Diabetes 2015;64:2859-2867
[4]. Laeger T, et al. J Clin Invest 2014;124:3913-3922
[5]. MarlattKL, et al. Curr Obes Rep 2017;6:389-396.
[6]. Kleinendorst L, et al. Exp Suppl 2019;111:419-441
[7]. Piaggi P, et al. J Endocrinol Invest 2018 Jan;41(1):83-89
[8]. Hall KD and Guo J. Gastroenterology 2017;152:1718-1727
[9]. Chinag JYL and Ferrel JM. Annu Rev Nutr 2019 Aug 21;39:175-200
[10]. Hui S, et al. Nature 2017 Nov 2;551(7678):115-118
[11]. Gilissen J, et al. Pharmacol Ther 2016 Mar;159:56-65
[12]. Regard JB, et al. Cell 2008; 135(3): 561-71
[13]. Mills EL, et a. Nature 2018 Aug;560(7716):102-106
[14]. De Vadder, et al. Cell Metab 2016 Jul 12;24(1):151-7
[15]. Wang K, et al. Cell Repor 2019 Jan 2;26(1):222-235.e5
[16]. Keiran N, et al. Nat Immunol 2019 May;20(5):581-592
[17]. van Diepen JA, et al. Diabetologia 2017 Jul;60(7):1304-1313
[18]. Serena C, et al. ISMEJ 2018 Jun;12(7):1642-1657
[19]. Sadagopan N,et al. Am J Hyperth 2007 Nov;20(11):1209-15
[20]. Aguiar CJ, et al. Cell Commun Signal 2014 Dec 24;12:78
[21]. Thomas DD, et al. J Endocr Soc. 2019 Jul 24;3(9):1727-1747
[22]. Andreoli MF, et al. J Endocrinol 2019 Jun 1;241(3):R81-R96
[23]. Hochachka PW, et al. Eur J Appl Physiol Occup Physiol 1976 Sep 23;35(4):235-42
[24]. Correa PR, et al. J Hepatol 2007;47:262 -269
[25]. Schrauwen P and Hesselink MK. Diabetologia 2008;51:1694 -1697
[26]. Ceperuelo-Mallafré V, et al. Diabetes Care 2019 Oct;42(10):1956-1965
[27]. AahanaShrestha, et al. EurJNutr 2017;56:671 -681
[28]. Jocken JWE, et al. Front Endocrinol (Lausanne) 2017; 8: 372
[29]. Kim KN, et al. Nutrients 2019 Oct 18;11(10)
[30]. Marianna P, et al. Rev Endocr Metab Disord 2019 Dec 6
[31]. Sutton EF, et al. Cell Metab 2018 Jun 5;27(6):1212-1221.e3
[32]. Zubrzycki A, et al. J Physiol Pharmacol 2018 Oct;69(5)
[33]. Puchalska P, et al. Cell Metab 2017 25,262 -284
[34]. Patel S, et al. Cell Metab 2019; 29(3): 707e8-718e8
[35]. Newman JC, et al. Rev Nutr 2017; 37: 51-76
[36]. Fisher FM, et al. Rev Physiol 2016; 78: 223-41
[37]. Iman GC, et al. Cell Metab 2008; 8: 169-74
[38]. Imai SI, et al. NPJ Aging Mech Dis 2016; 2:16017
[39]. Lee HC, et al. Annu Rev Pharmacol Toxicol 2001; 41: 317-45
[40]. Sutton EF, et al. Cell Metab.2018 Jun 5;27(6):1212-1221.e3
[41]. Anton SD, et al. Obesity (Silver Spring) 2018; 26: 254-68
[42]. Kim K-H, et al. Cell Res 2017 Nov;27(11):1309 -26
[43]. Zarrinpar A, et al. Cell Metab. 2014; 20, 1006 -1017
[44]. Furusawa Y, et al. Seminars in immunopathology 2015; 37, 17 -25
[45]. Jangi S, et al. Nature communications 2015; 7, 12015
[46]. Meydani SN, et al. Aging 2016; 8, 1416 -1431
[47]. Cignarella F, et al. Cell Metab 2018 Jun 5;27(6):1222-1235.e6

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HEALTH RESEARCH PROJECT APPLICATION FORM
HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS

Summary of evidence:

Energy sensing metabolites are emerging as being crucial in the relationship between energy expenditure and intake. They are responsible for the proper integration and coordination of the biological network in cells, particularly the concentration of nutrients and intermediate metabolites, key elements in the pathogenesis of obesity and T2D.

OBJECTIVES

General integrated goal of the coordinated project:

To elucidate the role of succinate and other metabolites derived from the intestinal microbiota such as SCFAs, as energy sensing metabolites in the context of obesity and T2D.

Specific objectives of Subproject 1 (SP1)

1a. - To investigate whether IF is better than DCR in terms of metabolic improvement through the study of:

1) the dynamics of gastrointestinal hormones and energy sensing metabolites
2) the intestinal microbiome
3) variability on succinate and SCFAs MCFAs and BA after weight loss

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HEALTH RESEARCH PROJECT APPLICATION FORM
METHODOLOGY

Design, study subjects, variables, data collection and analysis, study limitations.

Task 1:

Human study protocol:

A pilot clinical trial in human study participants will be assayed. Participants for intermittent fasting (IF) will be asked to fast for 24 hours on two days of the week (2/5 protocol).

1A.-Design:

Utilizing a **randomized, cross-over design**, study participants (n=15) will consume either lifestyle recommendations for a healthy Mediterranean diet under a continued daily caloric restriction diet (**DCR**) or will undertake an intermittent (**IF)** protocol. **Each study period** will be **8 weeks** - **total** study period will be **16 weeks + a 4-week washout** period between dietary exposures. The study participants will be **adults** who have obesity with a Body Mass Index **(BMI)25 kg/m² and 40 kg/m²** and have no contraindications for intermittent fasting (see inclusion and exclusion criteria below).

DCR protocol is detailed in the annex

IF protocol is detailed in the annex

Setting of the study: Endocrinology Service of Hospital Universitary Joan XXIII and Clinical Trial Unit of the IISPV.

A call for participation to the study will be performed at the local media and social networks, according to the recommendations of the IISPV Ethics Committee (these calls have been already done for previous studies by our group with a good acceptance).

Inclusion criteria: a) White men and women between 18 and 65 years of age; b) BMI range between 25 and 40 kg/ m^2; c) Absence of underlying pathology in medical and physical examination, except for those related to excess weight; d) Signature of the informed consent for participation in the study.

Exclusion criteria: a) Serious systemic disease not related to obesity, such as cancer, kidney, or severe liver disease. b) Systemic diseases with intrinsic inflammatory activity (autoimmune diseases such as rheumatoid arthritis and asthma); c) Pregnancy and lactation; d) Vegetarians or subjects subjected to an irregular diet; e) Patients with severe eating disorders; f) Patients with clinical symptoms and signs of infection in the previous month; g) Patients with chronic anti-inflammatory steroid treatments and/or nonsteroidal anti-inflammatory drugs; h) Recent antibiotic treatment; i) Psychiatric history; j) Uncontrolled alcoholism or drug abuse.

Sample size:

See annex

1A.a- Procedure:

For the IF protocol:

Subjects will meet a registered dietitian at the beginning of the study and every two weeks to learn how to follow the IF regimen at home. During each counseling session, the dietitian will work with the subject to develop individualized fast day meal plans. These plans will include menus, portion sizes and food lists that can be consistentwith their food preferences and prescribed calorie levels for the fast day. During these sessions, subjects will also be instructed as to how to make healthy food choices on the ad libitum food intake days.

For the DCR protocol:

Subjects will meet a registered dietitian at the beginning of the study and every two weeks to learn how to prepare and follow an adequate caloric restriction diet. During each counseling session, the dietitian will work with the subject to develop individualized meal plans. These plans will include menus, portion sizes, and food lists that are consistent with their food preferences and prescribed calorie levels for the rest of the week.

1A.b-Adherence to the IF diet:

Subjects will be asked to report any extra food item consumed on the fast day that did not comply with their prescribed plan by using the extra food record (*efr*). The *efr* will be reviewed by the study dietitian at each visit. If the *efr* indicates that the subject ate an extra food item on a fast day, that day will be labelled as “not adherent”. If

the *efr* reveals that the subject did not eat any extra food item, that day will be labelled as "adherent". Adherence data will be assessed each week as 1) total adherence (number of days adherent with diet) and 2) percentage adherence calculated by applying the following formula:
% Adherence = no. fast days adherent/ no. fast days in week x 100.

1A.c-Clinical/anthropometrical and analytical variables:

Routine clinical and analytical evaluation: (blood count, routine biochemistry, family and personal history, anthropometric measurements (weight, height, waist and hip circumference), frequency of food (previous week) questionnaires, and chronic treatments if any. The evaluation of physical activity and quality of life will be recorded according to validated questionnaires.

1B.-Analytical and functional test variables:

The degree of insulin resistance will be calculated according to the formula: HOMA-IR: [glucose (mmol / L) x insulin (mU / L)] / 22.5. Serum and plasma aliquots will be collected before and after each period of dietary intervention (for analytical determinations), and also stool samples for the study of microbiota.

1B.a-Metabolites and circulating factors:

Circulating succinate levels will be determined with EnzyChrom™ Succinate Assay Kit (BioAssay Systems, USA). Serum short chain fatty acids (SCFAs) including acetic (C_2), propionic (C_3), butyric (C_4) and formic acids will be analyzed by chromatography-mass spectrometry according to the methodology published by Lotti C et al [5]; Medium chain fatty acids (MCFAs) will also be quantified by chromatography in a separate run. Serum primary and secondary biliary acids (BA) will be quantified by the liquid chromatography -tandem mass spectrometry (LC/MS/ MS) method on the Ciberdem Metabolomics Platform http://www. iispv.cat/support_plataform/metabolomica. html.

1B.b-Meal Test and study of gastrointestinal hormones:

Before and after each restriction diet (DCR and IF diet) at the beginning ant at the end of each period (4 test). 200ml administration of Isosource Energy (Novartis, Switzerland) containing 398kcal (50% carbohydrates, 15% proteins and 35% lipids). Extraction at 0, 15, 30, 60 and 120 minutes.Determination of Plasma concentrations of glucose, insulin, GLP-1 andusing the Elisa kit (EZGLP1T-36K EMD Millipore, ThermoFisher) and succinate (EnzyChromTM Succinate Assay Kit (BioAssay Systems, USA)

1C-Microbial composition/metagenomic analysis:

Fresh stools will be collected before and after dietary intervention, which will be immediately frozen and stored at -80ºC until they are processed. Three days before sample collection, all patients will be placed on the same diet. They will be asked to complete a questionnaire. The microbial composition will be processed by Illumina Sequencing[6-9]. Briefly, we will obtain the microbial genomic DNA and the total RNA extraction; the sequencing of 16S rDNA and 16S rRNA; metagenomic sequencing; purification, amplification, and sequencing of mRNA; metagenomic and metatranscriptomics analysis [10]; protein extraction, separations and identification and data processing. All the sequences will be deposited in "the European Bioinformatics Institute database", with an access number. Metagenomic analysis will be done in collaboration with the team of Andres Moya from the Institute of Integrative Systems Biology at the Universitat de Valencia, with whom we actively collaborate.

Statistical analysis, Study limitations and contingency plan and References:

See annex

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HEALTH RESEARCH PROJECT APPLICATION FORM
STRATEGIC FRAMEWORK

(Please only use the space provided below)

1. **Project's capacity of approaching the objectives and priorities of the Societal Challenge of Health, Demographic Change and Well-being of the Spanish Strategy for Science, Technology and Innovation.**
2. **Relevance of the proposal for clinical-translational research.**

1. This project is part of the research oriented towards the challenges of society, whose goal is to improve the citizens' health encompassing the investigation of the most prevalent diseases. Specifically, the proposal focuses on two pathologies that are highly prevalent in the Spanish population: obesity and T2D. Both pathologies are in a phase of progressive increase of epidemic characteristics, (Prevalence of diabetes mellitus and impaired glucose regulation in Spain: the Di@bet.es Study. Diabetologia. 2012 Jan; 55 (1): 88-93 and Incidence of diabetes mellitus in Spain: results of the nation-wide cohort di@bet.es study. Scientific Reports. 2020 in press). Furthermore, theproposed research combines clinical aspects with biological process research related to the mechanisms involved in obesity and type 2 diabetes.

In this sense, the proposal submitted to this call may allow obtaining useful information in the field of Biotechnology with transfer capacity to clinical setting. Another relevant aspect outlined in the priorities of the Health challenge, is the development of personalized medicine, a relevant challenge that will result in benefits both for the patient, essentially, and for the national health system itself, optimizing therapies and individualizing the best treatment options in each case. Thus, the identification of predictive patterns of differential behavior of patients with T2D under metabolic surgery may help to simplify the choice of the surgical approach, according to a more consistent algorithm than the ones used so far. Previous data from our group, published in first line international journals, supports the validity of our hypothesis in this sense. Furthermore, the study in non-morbid obese patients helps us to cover a wide number of patients who usually ask clinicians for a good, efficacious, and safe dietary regimen to be healthier and feel better. The approach submitted will give us new clues about the efficacy of two common dietary protocols analysing new players in the weight loss mechanisms.

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HEALTH RESEARCH PROJECT APPLICATION FORM
ANNEXES

ANNEXES (TEXT) Max. 3 pages (15,700 characters)

The DCR protocol will be a daily reduction of 500 - 1000 kcal according to each individual's resting metabolic rate [1]; with a nutrient composition: < 30% of calories from fat, 15 - 25% from protein, 45 - 50% from carbohydrates [2].
The IF protocol will involve fasting for two days (non-consecutive) out of seven, with the fasting days separated by at least one day. A fasting day would entail the consumption of ~ 400-600 kcal at the evening meal (between 12,00 and 15,00 hours to ensure that each subject was undergoing the same duration of fasting), but no other caloric intake, and the participants can consume their desired intake on the other five days. The nutrient composition of the prescribed fasting day meal will be approximately: Total Fat: 13 g (60% mono or polyunsaturated fat), Protein 25g, Carbohydrate 60 g and Fiber 10 g. During fasting days, participants may consume water, tea or coffee (may contain milk max 10cc or cream, max 5 cc), unsweetened beverages such as iced coffee or tea, or a clear soup of vegetable broth.

Sample Size:
We have considered this trial as a pilot study. In this sense and following the recommendations on the calculation of the sample size for pilot studies [3], we have considered that a sample of at least 15 subjects per subproject (considering a dropout of 20% during follow up) will be sufficient to obtain valid exploratory data that allow us, in future, to carry out larger epidemiological studies [4].

Statistical analysis:
Strategy of the main statistical analysis: The data will be shown as percentages (categorical variables), means +/- standard deviation (continuous variables with normal distribution) or median with interquartile range (continuous variables with
non-normal distribution). The normality of the variables will be analysed with the Kolmogorov-Smirnoff test. To perform the Comparisons of each pre and post intervention group test for paired data will be used. To compare variables The McNemar test (paired data) will be used categorically / qualitatively. The continuous variables of normal distribution will be analysed with the Student t-test for paired data. For continuous variables that do not follow a normal distribution, first we will try to normalize by means of their logarithmic transformation to use parametric tests; if not, non-parametric tests for paired data (Wilcoxon T) will be used. The increase in concentrations of GLP-1 and succinate in response to dynamic functional tests will be measured by calculating the area under the curve. The correlations between the variables will be analysed with the Pearson or Spearman correlation coefficients, when appropriate. Multivariate analysis will be used to predict the clinical and/or functional variables that determine the improvement of clinical and metabolic variables after each diet protocol. Possible confusing variables or effect modifiers (interaction) will be selected based on clinical criteria (data from previous literature) and in function of the result of the univariant analysis ($p < 0.20$). All calculations will be performed with the STATA software package (v13.1 for Mac; StataCorp LP, College Station, Texas) and a value of bilateral $p < 0.05$ willbe used as significant.

References of methodology:

[1]. Flack KD, et al. J Acad Nutr Diet 2016 Sep;116(9):1413-1422

[2]. Goday A, et al. Nutr Diabetes 2016; 6: e230

[3]. Hertzog MA, et al. Res Nurs Health 2008; 31:180-91

[4]. Kimmelman J, et al. PLoS biology 2014;12:e1001863

[5]. Lotti C, et al. Anal Bioanal Chem 2017 Sep; 409(23):5555-5567

[6]. Vinaixa M, et al. Plos One 2011;6:e29052

[7]. Perez-Cobas AE, et al. Gut 2013; 62: 1591-601

[8]. Cardona S, et al. BMC Microbiol 2012; 12:158

[9]. Santiago A, et al. BMC Microbiol 2014; 14:112

[10]. Nielson HB, et al. Nat Biotechnol 2014; 32:822-8

[11]. Pachón-Peña G, et al. Stem Cells Transl Med 2016 Apr;5(4):464-75

[12]. Cheng CW, et al. Cell 2017 Feb 23;168(5):775-788.e12

[13]. Davidson MD and Khetani SR. Toxicol Sci 2020 Jan 16. pii: kfaa003

[14]. Ejarque M, et al. Int J Obes (Lond) 2019 Jun;43(6):1256-1268

[15]. Inder MV. Proc Natl Acad Sci USA 2012 Aug 21; 109(34): 13469