Title:

Effect of oral L-arginine 3.32 g a day on oxidative stress influencing beta cell function and insulin resistance. A phase 3, randomized, double-blind, placebo-controlled explorative study in overweight and obese patients with pre-diabetes.

Acronym Protocol: L-BIOARG

Versione: 2.0
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Promoter	**DAMOR farmaceutici** **Corso Milano, 46, 20900 Monza MB**

SIGNATURE PAGE

I declare that this protocol has been read carefully and fully understood. I agree to follow the study procedures as described in this protocol in accordance with Good Clinical Practice and all other regulatory requirements.

Prof. Andrea Giustina		4/11/2024
Primario U.O. per conto del Promotore/ Sperimentatore Principale	**Firma**	**Data**

INTRODUCTION

Insulin resistance and its mechanisms

Insulin resistance (IR) is a complex condition characterized by impaired insulin ability to activate glucose transport in various tissues, as well as an inability to suppress glucose production in the liver through gluconeogenesis (*Zhao et al., 2023*). This phenomenon extends beyond muscle cells and adipose tissue, affecting the kidney, gastrointestinal tract, brain, vascular system, and the pancreas endocrine function (*DeFronzo et al., 2015*). In healthy conditions, during fasting, the liver releases glucose to supply peripheral tissues, including the central nervous system, while adipocytes release free fatty acids and glycerol for muscle energy and hepatic NADH production. However, a meal stimulates insulin secretion by pancreatic β-cells, inhibiting lipolysis and gluconeogenesis, facilitating glucose uptake in muscle and adipose tissue. The majority of ingested glucose is stored in muscles as glycogen, while fraction of glucose accumulates in adipocytes to generate glycerol 3-phosphate for triglyceride synthesis. The liver absorbs glucose and becomes a substrate for glycogen synthesis under insulin stimulation, also initiating de novo lipogenesis using products from peripheral fasting catabolism (*James et al., 2021*).

At the molecular level, insulin binds to its receptor (INSR), leading to the phosphorylation of insulin receptor substrates (IRS), notably IRS1 and IRS2. This activation triggers intracellular signalling pathways, such as PI3K, which increases the expression of glucose transporter GLUT4 and inhibits transcription factor FOXO1, promoting glucose uptake and suppressing hepatic glucose production (*DeFronzo et al., 2015*). PI3K activation is a key mediator of insulin sensitivity, and alterations in this pathway are responsible for insulin resistance (*DeFronzo et al., 2015; James et al., 2021; Zhao et al., 2023).* Excessive phosphorylation of IRS proteins appears to be a contributing factor, inhibiting the phosphorylation of insulin receptor-associated tyrosine dimers (*Bouzakri et al., 2006; Copps & White, 2012).* Additionally, excessive serine-phosphorylation of IRS can lead to their degradation, reducing their availability as substrates for the receptor (*Hiratani et al., 2005*). These processes collectively lead to reduced receptor activation in response to insulin, with causes including ectopic lipid accumulation, mitochondrial dysfunction, hypoxia, inflammation, lipotoxicity, and endoplasmic reticulum stress (*DeFronzo et al., 2015; James et al., 2021; Zhao et al., 2023*).

These mechanisms are often present in conditions like obesity and type 2 diabetes, which are closely linked to IR. Indeed, IR is a risk factor for various metabolic diseases, including type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease, certain cancers, neurodegenerative diseases and frailty (*Zhao et al., 2023*). However, it's important to note that IR alone is not sufficient to cause type 2 diabetes; β-cell failure, responsible for insulin synthesis and secretion, is another critical factor. Multiple factors contribute to β-cell failure, including aging, genetic predisposition, resistance to or deficiency of incretins like GLP-1 and

GIP, lipotoxicity, glucotoxicity, hypersecretion of Islet Amyloid Polypeptide (IAPP), oxidative stress, and inflammation (*DeFronzo et al., 2015*).

Inflammation and oxidative stress

Insulin resistance, inflammation and oxidative stress are highly related to metabolic syndrome (MetS) and visceral obesity. Indeed, these two conditions are linked with chronic low-grade systemic inflammation, characterized by a considerable increase in the systemic levels of cytokines and adipo-cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and Leptin. Increased TNF- α levels and IL-6 levels are associated with insulin resistance mainly through the impairment of hepatic signalling and affection of IRS1 phosphorylation and GLUT-4 activity. Moreover, the impaired Leptin/Adiponectin ratio observed in obesity and metabolic syndrome is another well-known factor inducing insulin resistance. Furthermore, a synergistic relationship between low-grade systemic inflammation and oxidative stress is well-documented. Cytokines act as recruitment factors for immune cells along with adhesion molecules leading to their migration in the extracellular and subendothelial space. Here cytokines and immune cells are able to trigger the production of reactive oxygen (ROS) and nitrogen species (RNS) to cope with defense activities. On the opposite, persistent and unbalanced cytokine release results in increased pro-oxidative state (*Colak et al, J Med. Biochem., 2021*).

The reactive species can be free radicals and non-radical oxidants. The free radicals are unstable because of unpaired electrons presence in their outer electron orbit. Since free radicals are highly unstable and reactive, tend to neutralize themselves by reacting with other molecules causing their oxidation. Therefore, by reacting with important biological molecules, they can cause damage to lipids, proteins and DNA. Indeed, free radicals can induce cell membranes lipid peroxidation, DNA bonds ruptures and protein modifications, as the unfolding or alteration of protein structure, modification of key enzymes or their regulatory sites. These events are crucial for cells survival and usually trigger cell signalling alteration and programmed cell death pathways (*Cooke et al., The FASEB J, 2003; Vona R., Antioxidants, 2021*).

Superoxide, hydrogen peroxide and hydroxyl radical are the major components of ROS, generated in many biological processes. Superoxide is very toxic as it can act as a precursor to other ROS, such as hydrogen peroxide and the hydroxyl radical, leading to biological molecules damage (*Li et al., Gene, 2016).* Specifically, superoxide plays a key role in the initiation of lipid peroxidation. (*Fang et al. Nutrition, 2002).* Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) represent the first endogenous antioxidant defense against oxidative stress. SOD plays an important role in protecting from the toxic effects of superoxide radical through production of hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). CAT is an enzyme responsible for the removal of hydrogen peroxide preventing from the oxidative-stress-related damage (*Demirci-Çekiç et al, J. of Pharm. and Biomed. Analysis, 2021*). Glutathione (GSH) is one of the most important antioxidant molecules. GSH

directly reacts with ROS and other reactive species resulting in the formation of thiyl radicals (GS•), leading to different products such as glutathione disulfide (GSSG), mixed disulfides or glutathione sulphonamide. GSH is mainly used as a co-substrate by GPx reducing hydrogen peroxide or lipid peroxides, such as malonic dialdehyde (MDA) and 4-hydroxy-2-nonenal, with the production of glutathione disulfide (GSSG), water and alcohols. Both GSH and GSSG are substrates for the extracellular membrane-bound enzyme γ-L-Glutamyl transpeptidase (GGT) which catalyzes the production of L-glutamate and L-cysteinyl-glycine, providing the basis for excreted GSH and GSSG recycling by the cells. Upregulation of this process provides an additional mechanism for GSH maintenance, enhancing cellular antioxidant potential. (*Lutshack, J. of Amino Acids, 2012*).

Oxidative stress leads to endothelial dysfunction, reducing vasodilators bioavailability, especially NO, activating endothelial cells and providing a pro-inflammatory, proliferative and pro-coagulant state. This condition favours LDL-oxidation, immune cells recruitment and atherogenensis (*Hadi et al., Vasc. Health Risk Manage., 2005*). Oxidative stress is a well-known contributor to β-cells damage and progressive destruction (*Demirci-Çekiç et al, J of Pharm. and Biomed. Analysis, 2021)*. Many studies have described the association between pro-oxidant status and β-cells function *(R. Alghazeer, J. Med, Biomed. Sci., 2018; P. Codoñer-Franch, Pediatr. Diabetes, 2011).* Moreover, excess free fatty acids (FFAs) are always present in MetS due to insulin resistance and increased lipolysis. FFAs accumulate in extra-adipose tissues such as the liver and the muscle. In this condition a higher concentration of FFAs is delivered from mitochondria leading to excessive production of superoxide anion through an increased β-oxidation rate, impaired switching to carbohydrate substrate, decreased tricarboxylic acid cycle activity and impaired mitochondrial electron transport chain. Increased β-oxidation increases mitochondrial NADH/NAD+ ratio, resulting in increased activation of protein kinase C (PKC), advanced glycosylation end products (AGEs) and NF-kB. All these mechanisms contribute to ROS production, nitric oxide synthase (eNOS) inhibition in endothelial cells, reduction in nitric oxide (NO) production in vascular smooth muscle cells and to the activation of nuclear transcription factor NF-kB maintaining and worsening inflammation pathways (*Colak et al, J. Med. Biochem., 2009).*

L-arginine

L-arginine, a conditionally essential amino acid, plays a crucial role in the production of Nitric Oxide (NO). Under normal conditions, L-arginine is metabolized by NOS, producing NO and L-citrulline, which have favorable effects on endothelial function, cardiovascular health, insulin secretion, and sensitivity. L-arginine is also involved in the synthesis of various essential compounds and has diverse functions, including promoting growth hormone secretion, T cell proliferation, and immune responses (*Forzano et al., 2023*). Several preclinical and clinical studies have demonstrated the beneficial effects of L-arginine supplementation in insulin-resistant, prediabetic, and diabetic patients. It has a protective role on β-cell function and neogenesis, as well as improving insulin sensitivity in obese and type 2 diabetic patients. One study has shown that L-arginine supplementation enhances insulin secretion through a GLP-1 mediated mechanism (*Clemmensen C, Endocrinology, 2013*). Additionally, L-arginine

supplementation has been reported to affect adiposity by reducing white adipose tissue in favour of brown adipose tissue (*Shi XY, Endocrinology, 2008*). Importantly, long-term follow-up studies suggest that L-arginine supplementation may significantly reduce the cumulative incidence of type 2 diabetes in high-risk individuals with impaired glucose tolerance and metabolic syndrome (*Monti L et al., Eur J Nutr 2018*).

Furthermore, L-arginine has been reported to reduce oxidative stress and improve endothelial function leading to the production of NO, stimulating glutathione reductase and glutathione (GSH) de novo synthesis and enhancing antioxidant enzymes transcription against oxidative stress. A study on rats by Liang et al. (*Liang et al., Food and Chem. Toxicology, 2015*) demonstrated the enhancement of nuclear factor erythroid 2(NF-E2)-related factor 2 (Nrf2), a key transcription factor which positively regulates GSH metabolism and promotes the cellular defense mechanisms against oxidative stress through inducing and up-regulating superoxide dismutase (SOD) and catalase (CAT) gene expressions and other molecules involved in antioxidant pathways. GSH is a tripeptide constituted by L-glutamate, L-cysteine, and L-glycine. GSH synthesis depends also upon nutritional intake. L-Arginine is a well-established substrate for glutamate synthesis. (*Bansal and Ochoa, 2003; Cynober et al., 1995; Morris, 2004, 2006; Wu and Morris, 1998*). Farther, Liang et al (*Liang et al., Food and Chem. Toxicology, 2015*) described a positive correlation between hepatic GSH content and arginase expression in rats, highly suggesting L-Arginine as one of the precursors in GSH synthesis. This evidence highlights the role of L-Arginine not only as a mere NO donator but as a possible key part involved in prooxidant-antioxidant balance.

Side effects

No side effects have been reported in previously conducted studies.

Rationale

Recent studies have demonstrated beneficial effects of the use of L-arginine supplementation as a nutrient treatment in diabetes and prediabetes, with documented beneficial effects on β-cell function (*Mendez JD, Biomed Pharmacol., 2005; Vasilijevic A, J Physiol. 2007*) and insulin sensitivity (*Wascher TC, Eur J Clin Investig, 1997; Lucotti P, Metabolism, 2009*).

A long-term follow-up randomized clinical trial also demonstrated a reduced cumulative incidence of type 2 diabetes among subjects at high risk of developing the disease (*Monti L et al., Eur J Nutr, 2018*). The same study reported a significantly AOPP (Advanced oxidation protein products) levels reduction at 18 months in L-arginine arm compared to placebo and this effect was maintained through the 108 months of follow up. Indeed, AOPP levels decreased by 26.1% at 18 months, remaining lower during the post intervention period in L-arginine group (Figure1. AOPP at 108 months: 773.2 ± 244.4 vs. 1045.7 ± 282.9 μmol/l, $p < 0.05$) (*Monti L et al., Eur J Nutr, 2018*).

However, in this study, AOPP levels were firstly evaluated at baseline and then only 18 months after starting treatment. Therefore, to date, no data are currently available regarding the effects of a short-term treatment on AOPPs levels. In addition, data in vivo on rats suggest a rapid improvement in antioxidant defense and in total antioxidative capacity in plasma and liver after

a 14 days treatment period with L-arginine (Liang et al., Food and Chem. Toxicology, 2015).

Advanced oxidation protein products are formed during oxidative stress by myeloperoxidase action in activated neutrophils through chloraminated oxidants production. AOPPs molecular structure is similar to advanced glycation endproducts (AGEs) as they have similar biological activities. Precisely, AOPPs exert induction of proinflammatory cytokines and adhesion molecules maintaining inflammatory pathways activated and favoring pro-oxidant state (*Witko-Sarsat V, Kidney Int 1996; Catakay U, Diabetes Metab 2005*). In addition, many reports highlighted a strict correlation between AOPPs and glucose metabolism (*Kalousová M, Physiol Res 2002; Martín-Gallán P, Free Radic Biol Med 2003; Piwowar A, Diabetes Res Clin Pract, 2007;*) suggesting being an early marker of diabetes mellitus and metabolic syndrome (*Zurawska-Plaksej E, J Endocrinol Invest., 2014; Martín-Gallán P, Free Radic Biol Med 2003*). The prooxidant-antioxidant index (PAI) has been proposed as possible marker of MetS. It is expressed as the ratio between the AOPP levels and the total radical-trapping antioxidant capacity (TRAP), a surrogate for the total antioxidant defenses in the plasma. An interesting study by Venturini et al. evidenced the relationship between AOPPs and MetS also highlighting that PAI progressively increased ($P < 0.05$) according to the number of MetS components, whereas AOPPs and total radical-trapping antioxidant parameter increased ($P < 0.05$) when 5 components were compared with 3 and 4 components (*Venturini et al., Nutr Research, 2015*).

Lipid peroxidation products such as malondialdehyde (MDA), and 4-hydroxynonenal (HNE) have been reported to be highly related to inflammation and MetS. These molecules are considered important biomarkers of oxidative stress because of the high susceptibility of polyunsaturated fatty acids-rich cell membranes to lipid peroxidation when exposed to ROS or other free radicals (*Nair, Free Radic Biol Med, 2007; Demirci-Çekiç et al, J of Pharm. and Biomed. Analysis, 2021*). A positive correlation has been described by several studies among obesity, IR, MetS, glycaemic control and MDA and HNE levels (*Srikanthan, Molecules, 2016, Picklo MJ, Nutr Rev 2015*).

Regarding the antioxidant enzymes, a study by Lang et al. on rats fed with L-arginine for 14 days demonstrated a significant stimulation in GSH synthesis enzymes, CAT, SOD and GPx activity and mRNA expression enhancing the total antioxidative capacity in plasma (T-AOC or TRAP) and in the liver strongly suggesting that L-arginine oral administration can ameliorate antioxidant defenses (*Liang et al., Food and Chem. Toxicology, 2015*).

To conduct this study, we selected a L-arginine dosage of 3,32 g/day, based on evidence that this dosage was associated to an improvement of the inflammatory response and beneficial modulating of oxidative stress in favor of an antioxidant state (*G. Fiorentino et al., Eclinicalmedicine, 2021*).

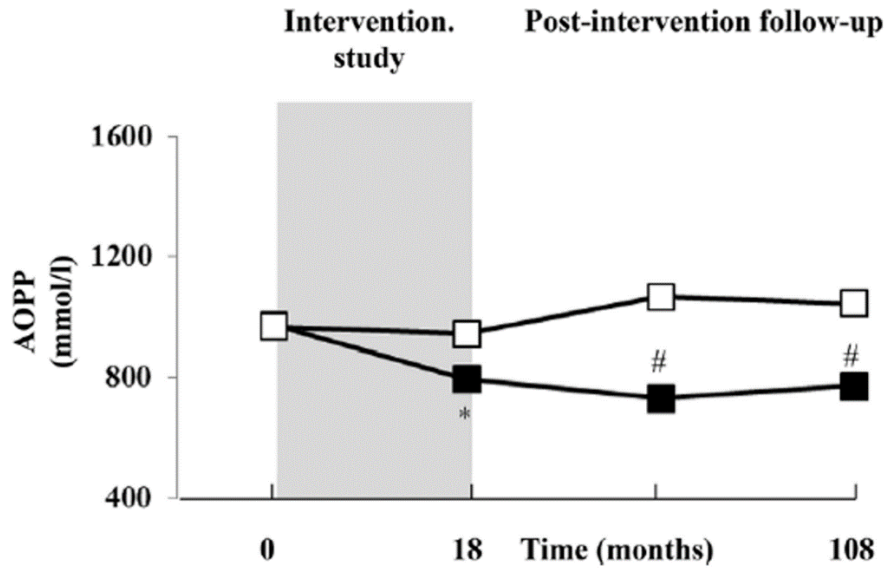

Figure1: Mean AOPP levels in the L-arginine (black boxes) and placebo (white boxes) groups; *$p < 0.05$ vs. placebo; #$p < 0.01$ vs. placebo.
Adapted from *Monti L et al., Eur J Nutr, 2018.*

Objective

The objective of this clinical trial is to explore whether L-arginine supplementation with 3.32g/day may enhance antioxidant defenses in overweight and obese patients with pre-diabetes and metabolic syndrome, possibly providing beta cell function and insulin resistance improvement. The primary efficacy endpoint will be to explore the effects of a short-term L-arginine supplementation on AOPP levels and PAI after three months of treatment, based on the rationale explained above. Secondary, the effects of L-arginine supplementation on lipid peroxidation products and antioxidant defenses will be explored. Tertiary, the effects of L-arginine supplementation on insulin resistance and glucose metabolism parameters will be also evaluated.

METHODS

Study design

The study is a prospective, double-blind, placebo-controlled single-center, phase 3 study. It will involve 42 patients with BMI ≥25 kg/m^2 and pre-diabetes. They will all receive a calorie-restricted Mediterranean diet, a physical activity program, and will be randomly (1:1) assigned to either L-arginine 1.66 g twice a day for 90 consecutive days (treatment group) or placebo (control group). To record eating and exercise habits, a food and physical activity diary will be kept by the patient throughout the study period. An individualized nutrition regimen will be prescribed to the patient based on characteristics like gender, age, BMI, physical activity and preferences.

Patients undergoing metabolic assessment at the Endocrinology Unit of IRCCS Ospedale San Raffaele, willing to participate in the trial and meeting the inclusion criteria, will be enrolled in this study. Patients will be screened at our "dysmetabolic diseases" outpatient clinic. The patients with Metabolic Syndrome according to WHO definition and with BMI ≥25 kg/m^2, glycemia between 100 mg/dl and 125 mg/dl and/or HbA1c between 5.7% and 6.4%, according to ADA definition of pre-diabetes status *(ADA Standards of Care 2023)* will be enrolled and subsequently randomized during the **baseline study visit**. During this visit, the participants will undergo evaluation of anthropometric measures, in accordance with standard clinical practices, including weight, height, waist circumference, and waist-hip ratio. Furthermore, 1600 meal plan and physical activity will be evaluated. Additionally, blood samples will be collected for AOPP, TBARs, TRAP, total glutathione, Catalase and SOD activity, urate, IL-6, TNF-a, VES, CRP, total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides, blood glucose, insulin, C-peptide levels and glycated hemoglobin (HbA1c) measurement at the central laboratory of IRCCS Ospedale San Raffaele (measured through standardized HPLC technique (TOSOH HLC723®-G11 Analyzer, Tosoh Europe NV). Patients will be randomized to receive L-bioarginine or placebo for 90 days. The follow-up visit will be performed within 1 and 5 days after the last dose of study treatment. At day 91-95 a **Visit 1** will be performed to collect the same parameters collected at the Baseline study visit and the patient will undergo a second blood sample collection for the same parameters determined at baseline visit.

Sample size

The main endpoint of the study is to analyze the differences in the reduction of AOPP levels in patients treated with L-arginine versus placebo. Based on results of a previous study (*Monti L et al., Eur J Nutr 2018*) showing a reduction of AOPP levels in patients treated with L-arginine by 20-30% as compared to negligible effect in the placebo group, for the present study we hypothesized that 50% of patients treated with L-arginine will achieve a reduction of at least 20% in AOPP levels compared to only 10% (or less) of patients in the placebo group. Based on these assumptions, 17 patients in each group would be needed to obtain a statistical power of 80% with $\alpha = 0.05$ (two tailed). Estimating a drop-out rate of about 10%, the total number of enrolled patients will be 40 (20 in each group).

Number of patients: 40 patients with BMI ≥25 kg/m^2, pre-diabetes or MetS will be enrolled and randomized in 2 groups, i.e. 20 treated with L-arginine supplementation 1.66 g twice a day and 20 with placebo for 90 consecutive days.

Study period	Projected starting date (first-patient-in):	December 2024
	Projected completion of patient accrual (last-patient-in):	December 2025
	Projected study end date (last-patient-last-visit):	March 2026

Eligibility criteria

Inclusion criteria: consented male and female patients aged 20-70 years with Body Mass Index

(BMI) ≥25 kg/m^2, pre-diabetes (fasting glucose 100-125 mg/dL and HbA1c 5.7-6.5%) or metabolic syndrome, defined according to modified NCEP-ATP III criteria (*Grundy SM et al., Circulation, 2005*) as the presence of three or more of the following clinical features: blood glucose levels >100 mg/dL, HDL-cholesterol <40 mg/dL in males and <50 mg/dL in females, triglycerides levels >150 mg/dL, waist circumference >102 cm in males and >88 cm in females and hypertension, defined as repeated blood pressure measurements >130/85 mmHg.

Exclusion criteria: 1. moderate to severe renal impairment (calculated creatinine clearance (CrCl) <60 mL/min according to the Cockcroft-Gault formula); 2. hepatic dysfunction (ALT/AST >3 x upper limit of normal and total bilirubin >3 mg/dL); 3. hypoalbuminemia (serum albumin <3 g/dL); 4. history of any past or current clinically significant cardiovascular diseases; 5. monogenic, secondary and pharmacological causes of diabetes and obesity; 6. any other clinical condition/disease that the Principal Investigator believes might confound study outcome; 7. patients on treatment with insulin or any anti-diabetic drugs or medications known to influence glucose tolerance will also be excluded; 8. pregnant or breast-feeding women.

Efficacy endpoints

Efficacy endpoints will be:

- Changes from the baseline in AOPPs, TRAP (Total plasma radical-trapping antioxidant capacity) as a measure of all the hydrosoluble and the liposoluble antioxidants present in plasma (Primary endpoints) and PAI (Prooxidant-antioxidant index), calculated as AOPPs (µmol/L) divided by TRAP (µM Trolox). These are the primary composite endpoint;
- TBARs quantitative assays as a measure of oxidative stress and lipid peroxidation products (Secondary endpoint);
- Catalase (CAT) and Superoxide-dismutase (SOD) quantitative assays (Secondary endpoint);
- Total glutathione quantitative assay and GSH/GSSH ratio (Secondary endpoint);
- Serum IL-6, TNF-a, VES, CRP, GGT, urate, total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides (Tertiary endpoint);
- HbA1c (mmol/mol) (Tertiary endpoint);
- Proportion of patients with Impaired fasting glucose (IFG) (Tertiary endpoint);
- Changes in Body weight and BMI (Tertiary endpoint);
- Abdominal obesity (waist and waist-hip ratio circumference, measured by the same operator) (Tertiary endpoint);

- HOMA-B%, as a simplified one-sample β-cells function assessment model, calculated by the following formula equation:

$$\frac{(\boldsymbol{fasting\ insulin\ x\ 20})}{(\boldsymbol{fasting\ glucose\ -\ 3.5})}$$

with glucose expressed in mmol/L and insulin mU/l) *(Matthews et al., 1985)* (Tertiary endpoint);

- HOMA-IR, as a simplified one-sample insulin-resistance assessment model, calculated by the following formula equation:

$$\frac{(\boldsymbol{fasting\ blood\ glucose\ x\ fasting\ insulinemia}}{\boldsymbol{22.5}}$$

with glucose expressed in mmol/L and insulin mU/l (normal range 0.23-2.5) *(Matthews et al., 1985)* (Tertiary endpoint).

Patient management and treatment

Patients enrolled in the study will be randomized to receive either L-arginine treatment or placebo. Randomization will be performed through an excel file to favor the allocation concealment. Indeed, the company will provide clinicians with an excel table showing preparations A and B by patient numbers ensuring randomization using the program's "random" function (see attached).
Blindness of the study is ensured because Farmaceutici Damor will provide the hospital with identical packages of vials of the preparation under study distinguished only by the indication "preparation A" and "preparation B." The vials will be identical in both aesthetics and palatability (see attached data sheets).
Only the company will be aware of which of the two preparations corresponds to Bioarginina®zero and which to placebo.
It is remarkable that L-arginine treatment is already commercially available and although no notable adverse events have been reported, there are limited and insufficient data regarding its efficacy in improving clinical parameters concerning insulin resistance and beta- cell function. For this reason, both treatments (L-arginine vs. placebo) can be considered ethically accepted in this clinical setting. The two groups will be managed with the same modalities and their management will be carried out as part of standard clinical practice visits.

Sides effects management

No side effects are expected for this study as the L-arginine was not reported to induce side effects. The occurrence of side effects will be monitored on an ongoing basis and reported with narratives, allowing for the collection of additional information, as warranted.

Description of data management and statistical analysis

Data will be extracted from the electronic patient data management system and will be obtained through medical interviews and visits at our outpatient clinic. Data will be analyzed using descriptive statistics for all study variables. Categorical variables will be summarized as counts and percentages. The normality of continuous variables will be assessed using the Kolmogorov–Smirnov test ($p > 0.05$). For normally distributed data, means and standard deviations (SD) will be reported. In cases where the data distribution deviates from normality, medians and inter-quartile ranges will be utilized. Statistical significance testing will be carried out using appropriate methods: Fisher exact test or $\chi 2$ test (for categorial variables) and Student T-test or Mann-Whitney test, (for continuous variables). These tests will be applied to discern significant differences in proportions, means, or medians between the two groups at baseline. Furthermore, paired samples T-test or Wilcoxon signed-rank test will be employed to evaluate changes from baseline to follow-up for all endpoints. Multiple logistic regression analyses will be used to estimate the effects of L-arginine treatment on the protocol efficacy endpoints evaluating the impact of the other covariates and variables collected in the study as the demographics and anthropometric data, or dietary and physical activity adherence (collected as binary categorical variables).
A p-value of <0.05 will be considered statistically significant. Statistical analysis will be conducted using IBM SPSS Statistics (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.).

ETHICAL ASPECTS AND DISSEMINATION

Ethical aspects and informed consent

This protocol, the information attached to the informed consent, the informed consent itself, the privacy protection information, and the privacy consent have been submitted to the Territorial Ethics Committee of Lombardia (CET Lombardia). Subjects will be informed about the clinical study both in written and verbal form by physicians of the Endocrinology Unit of IRCCS Ospedale San Raffaele. Ample opportunity will be given to decide whether to participate in this study and to ask questions regarding the study. It will also be clearly explained that the patient's participation in the study can be discontinued at any time, without providing a reason, and that the patient will not be penalized in any way for this decision. All information in the informed consent will be communicated to patients in an understandable language. The physician conducting the study and the patient will personally sign and date the informed consent form with a declaration regarding data confidentiality. The informed consent will be stored in accordance with Good Clinical Practice (GCP) standards, current regulations, and in line with OSR internal policies. A copy will be given to the patient.

The research project will be conducted in accordance with national and international norms and legislation, as well as European standards of research ethics, as expressed in the applicable legislation/norms:

- The Declaration of Helsinki;
- Ethical principles for medical research involving human subjects;
- Council of Europe Convention on Human Rights and Biomedicine (Oviedo Bioethics Convention);
- Directive 95/46/EC (amended 2003) and Regulation (EC) No 45/2001 of the European Parliament and of the Council, dated December 18, 2001, on the protection of individuals concerning the processing of personal data;
- National legislation and regulations applicable to the specific research activity: Legislative Decree No. 158/2012; Ministerial Decree 8/2/2013 published in the Official Gazette No. 96 of 24/4/2013; Regional Decree of Lombardy No. 5493 of 25/6/2013; Chapter on fundamental rights of the EU.

Procedures for the collection, storage, protection, preservation, and destruction of sensitive data include:

- Obtaining informed consent;
- Confidentiality of personal data;
- Measures for the coding and storage of samples;

This study will be financially supported by the DAMOR Farmaceutici. The study will be covered by a specific insurance policy, undersigned by the sponsor.

Data confidentiality

Guaranteed according to current regulations. Data will be processed using computerized and non-computerized tools and stored in paper archives and electronic databases and will be disseminated only in strictly anonymous form, for example through scientific publications, statistics and scientific conferences. Only the medical staff of the Endocrinology Unit will be aware of patient personal data. In addition, the Ethics Committee and the Italian Health Authorities will have access to the personal data of patients enrolled in this study, including information contained in original medical documentation, exclusively for the purposes of checking the procedures of the Practice and the correctness and accuracy of the data collected. In any case, all precautions to ensure the necessary confidentiality of patients identity within the limits set by applicable regulations, in particular in strict compliance with the principles of correctness, competence, accuracy, relevance and completeness of treatment (art.11 D.Lgs. 196/2003 - Code for the protection of personal data).

Data ownership and methods of publication

The sponsor of this study declares that all data from this study belong to the sponsor. No specific rule is established for the publication of study data, which is intended to be disseminated anonymously at scientific congresses and in publications in journals.