Graduate School in Biochemical, Nutritional and Metabolic Sciences

UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI AGRARIA

L-ARGININE IN HEALTHY FOOD IN OBESE SUBJECTS WITH IMPAIRED GLUCOSE TOLERANCE AND METABOLIC SYNDROME

Elena Galluccio

2012/2013

INDEX

RIASSUNTO

Introduzione: La sindrome metabolica è un cluster di anomalie metaboliche, che comprende insulino-resistenza, alterata regolazione del glucosio, ipertensione, dislipidemia, obesità e aumentato rischio di malattia cardiovascolare. L'insulino-resistenza (IR) è stata collegata ad alterati indici di rigenerazione vascolare, infatti, molti dati mettono in relazione la resistenza insulinica al ridotto numero e funzionalità delle cellule endoteliali progenitrici (EPC). Fra i molteplici meccanismi molecolari implicati vi sono la riduzione dell'attività e biodisponibilità dell'ossido nitrico (NO) e l'aumento dello stress ossidativo. Questi aspetti sono legati sia all'anormale attività/produzione di fattori di crescita e chemochine che all'alterato signaling intracellulare. L'insulino-resistenza altera la via PI3K/Akt/eNOS stimolata dall'insulina che sappiamo essere coinvolta nell'angiogenesi, giocando un ruolo importante nella riparazione del danno vascolare. Infatti, è stato dimostrato che l'IR, attraverso la down regulation della via del segnale PI3/Akt, causa un decremento della produzione di NO, il quale regola la motilità cellulare e la mobilizzazione delle cellule staminali dal midollo osseo. L'ossido nitrico di derivazione endoteliale, potente vasodilatatore endogeno, viene sintetizzato dall'enzima ossido nitrico sintetasi endoteliale in una reazione multistep a partire da L-arginina. È stato dimostrato che la L-arginina, potenzia l'uptake di glucosio mediato dall'insulina incrementando il flusso sanguigno. Alla luce dei dati ottenuti con la supplementazione orale di L-arginina, abbiamo pensato ad una diversa somministrazione di quest'amminoacido, sottoforma di prodotto alimentare, molto più gradito ai pazienti. Abbiamo pertanto creato un biscotto contenente L-arginina (10%), completamente biodisponibile (> 99%), con un basso contenuto di carboidrati. Il biscotto è stato ottenuto combinando L-arginina con fiocchi di cereali misti, riso soffiato, nocciole e scorza di arancia candita, mediante un processo innovativo di aggregazione (sotto brevetto) di assemblamento/sagomatura del prodotto. Ogni biscotto (circa 10 g) contiene almeno 1 g di L-arginina. Dati precedenti, ottenuti in uno studio pilota su soggetti sani, hanno mostrato che nel biscotto la

biodisponibilità di L-arginina è del 100% e che il prodotto alimentare ha un effetto benefico sulla funzione endoteliale e vascolare aumentando l'ossido nitrico e il suo secondo messaggero, il cGMP.

Scopo: Questo progetto è uno studio crossover in doppio cieco condotto su soggetti obesi con alterata tolleranza al glucosio (IGT) e sindrome metabolica (SM), al fine di valutare gli effetti della L-arginina contenuta nei biscotti, rispetto ai biscotti senza L-arginina, sulla funzione endoteliale, sul signalling insulinico e sulla mobilizzazione delle EPCs.

Disegno dello studio e metodi: Lo studio, durato sette settimane, è stato condotto su quindici soggetti obesi con IGT e MS e consisteva nel consumo giornaliero di 6 biscotti (arricchiti con L-arginina o placebo) divisi in due snack, mattina pomeriggio. Il peso e la massa corporea sono stati valutati con bioimpedenziometria. All'inizio e dopo ciascun periodo di intervento nutrizionale, sono stati eseguiti l'OGTT e prelievi ematici, a digiuno, per l'esecuzione di saggi biochimici e per la separazione di cellule endoteliali progenitrici (EPC). Il flusso ematico dell'avambraccio (FBF) e l'iperemia reattiva (vasodilatazione endotelio-dipendente) è stata misurata con la pletismografia a strain-gauge. Le EPC sono state valutate mediante citofluorimetria. L'espressione genica è stata valutata tramite PCR real time.

Risultati

Nel gruppo trattato con biscotti arricchiti con L-arginina è stata riscontrata una riduzione del peso corporeo, accompagnata da una significativa riduzione della massa grassa, con livelli di NOx, cGMP e del flusso sanguigno post-ischemico significativamente aumentati rispetto al gruppo trattato con biscotti placebo. Inoltre, il trattamento con L-arginina ha promosso un incremento sia dei livelli di espressione genica di eNOS e Akt (nelle EPC), sia dei livelli plasmatici di SDF-1α, VEGF-α, MMP-9, SCF, ed una riduzione dei livelli plasmatici di ADMA.

Conclusioni:

I risultati dello studio rivelano che il consumo di biscotti arricchiti con L-arginina, per 14 giorni é utile per migliorare la funzione endoteliale e vascolare, il metabolismo del glucosio, la diminuzione del peso e della massa grassa in soggetti con IGT e MS. La

forza di questo studio è che la perdita di peso riscontrata è stata ottenuta senza l'aiuto di un programma di attività fisica strutturata nei volontari sedentari. Inoltre è stato dimostrato che i biscotti arricchiti con L-arginina sono in grado di aumentare l'espressione genica di Akt e di eNOS e di migliorare la mobilizzazione e le proprietà di differenziazione cellulare, aumentando sia specifici marker periferici sia il numero di EPC circolanti.

ABSTRACT

Introduction: Metabolic syndrome is a cluster of metabolic abnormalities, which includes impaired glucose regulation, insulin resistance, hypertension, dyslipidemia, obesity and increased cardiovascular disease risk. Insulin resistance (IR) has been linked with altered indices of vascular regeneration. Abundant data also link the insulin resistance with reduced endothelial progenitor cell (EPCs) number and function. Among many molecular mechanisms involved, there are reduced nitric oxide (NO) bioavailability and oxidative stress, which are both linked with abnormal growth factor and chemokine activity and altered intracellular signaling. Insulin-stimulated PI3K/Akt/eNOS signaling, which is involved in angiogenesis, playing an important role in vascular repair, is impaired in insulin resistance. In fact it has been demonstrate that IR, through the down regulation of the signaling pathway PI3K/Akt, causes a decrease of NO production, which regulates cell motility and stem cells mobilization from bone marrow.

The endothelium-derived nitric oxide is a potent endogenous vasodilator that plays a major role in vascular tone; it is synthesized by the enzyme endothelial nitric oxide synthase (eNOS) in a multistep reaction from L-arginine. It has been previously demonstrated that L-arginine potentiates insulin-mediated glucose uptake by increasing blood flow.

In light of these data obtained with L-arginine oral supplementation, we considered a new way of administration of this aminoacid, that could be more acceptable by patients, through an innovative food product containing L-arginine (at least 10%),

completely bioavailable (>99%), with a low content of carbohydrates. Recently we developed a food product containing L-arginine, as a biscuit. L-arginine enriched biscuits were manufactured in a single batch by combining L-arginine with other ingredients (mixed cereal flakes, puffed rice, hazelnuts, candied orange peel) by means of an aggregation/shaping innovative process (under patenting) based on sonication. Each biscuit (about 10 g) contained at least 1 g of L-arginine. Previous data, obtained in a pilot postprandial study in healthy subjects, showed a quite complete bio-availability of L-arginine in the biscuit and beneficial effect on endothelial and vascular function by increasing nitric oxide and its second messenger, cGMP.

Aim: This project is a double-blind crossover study performed in obese subjects with impaired glucose tolerance (IGT) and metabolic syndrome (MS), in order to evaluate the effects of the L-arginine-enriched biscuits, compared with placebo biscuits, on endothelial function, insulin signalling and EPC mobilization.

Study design and methods: The study consisted of a 14-day randomized double-blind placebo-controlled crossover intervention trial in which 15 obese subjects with IGT and MS consumed 6 biscuits (enriched with L-arginine or placebo) divided into two snacks, in the morning and in the afternoon..At baseline and at the end of each treatment period blood samples were collected after an overnight fast and during OGTT for EPC separation and serum/plasma biochemistries and body weight and composition were evaluated by bioimpedenziometry. The forearm blood flow (FBF) and reactive hyperemia (endothelium-dependent vasodilation) were measured with strain-gauge plethysmography. EPCs were evaluated by flow cytometry. Gene expression was evaluate by real time PCR.

Results: In the group receiving L-arginine-enriched biscuits we found a body weight reduction, accompanied by a significant reduction in fat mass, higher L-arginine, NOx and cGMP levels and post-ischemic blood flow significantly increased compared with the group receiving placebo biscuits. Moreover L-arginine enhanced both eNOS and Akt mRNA expression levels (in EPCs) and SDF-1α, VEGF-α, MMP-9, SCF plasmatic levels, while reduced ADMA plasmatic levels.

Conclusion: The study results reveal that consumption of L-arginine-enriched biscuits for 14 days was safe and useful for improving endothelial and vascular function, glucose metabolism, decreasing body weight and fat mass in subjects with IGT and MS. The strength of the present study is that weight loss was achieved without the help of a structured physical activity program in the sedentary volunteers. Moreover, we demonstrated that the L-arginine-enriched biscuit was able to increase Akt and eNOS gene expression and to improve cell mobilization and differentiation properties, by increasing both the specific peripheral markers and the number of circulating EPCs.

1. PREFACE

1.1. METABOLIC SYNDROME

Definition and brief history

The metabolic syndrome is a heterogeneous clinical entity, represented by a cluster of multiple abnormalities, affecting obesity, especially abdominal obesity, insulin resistance, impaired glucose tolerance, dyslipidemia (high triglycerides and low HDL cholesterol) and high blood pressure. The prevalence of these alterations is different between the genders at different ages and in different ethnic groups. There are still few problems related to the definition and diagnosis of metabolic syndrome: the proposed components are continuous variables and as far as is necessary to establish a cut-off, there is not yet a consensus on the specific values to establish the diagnosis of each component; these variables are certainly interrelated, but the pathophysiology of their relationship is not yet clearly understood, and still debated whether or not to include insulin resistance and diabetes among the components of diagnostic and whether to include other variables (such as those related to inflammation or coagulation). It was called plurimetabolic syndrome (Avogaro P), metabolic syndrome (Haller H), syndrome X (Reaven GM), deadly quartet (Kaplan NM), insulin resistance syndrome (De Fronzo RA), The description of the metabolic syndrome and the attempt to standardize the diagnostic criteria have a history that dates back to the early '600, but that still keeps the lively debate. It was Nicolaes Tulp (1593-1674) first, in Amsterdam, to describe a case of syndrome hypertriglyceridemia (Erklens DW). Tulp established the link between hypertriglyceridemia and ingestion of saturated fatty acids ("pure milk in the blood"), obesity and bleeding tendency. Not only that, he suggested a therapeutic approach correctly indicating a reduction in the contribution of saturated fatty acids, he also recognized the association between early atherosclerosis and sudden death. About 250 years later, Morgagni described very clearly the association between visceral obesity, hypertension, hyperuricemia, atherosclerosis and obstructive sleep apnea

syndrome, long time before the metabolic syndrome and the syndrome of obstructive sleep apnea were defined (Morgagni GB). The frequent simultaneous presence of obesity, hyperlipidemia, diabetes and hypertension was described in 1977 by Haller et al., who first uses the term "metabolic syndrome" and describes the association with atherosclerosis (Haller H). Reaven in 1988 introduces the concept of Syndrome X to identify the group of disorders relating to the metabolism of glucose and insulin, dyslipidemia and hypertension. Reaven suggested that insulin resistance, which causes hyperinsulinemia, characterizes this group of disorders and is an important cardiovascular risk factor in itself (Reaven GM). It should be noted that Reaven, in his description, had not included among the components of the syndrome overweight and/or obesity. In 1991, Ferrannini et al. also suggested that this set of disorders was determined by insulin resistance and use the term "insulin resistance syndrome" (Ferrannini E). A definition of the metabolic syndrome was given by the "WHO Working Group on diabetes" in 1998, then amended in 1999, with a list of criteria for clinical diagnosis.

There were different criteria for the definition of metabolic syndrome, in 2001, "United States National Cholesterol Education Program Adult Treatment Panel III (ATPIII) report" proposed a set of criteria similar to those proposed by the WHO, except for the fact that the key component of visceral obesity and insulin instead resistance. The ATP III does not find enough evidence to recommend the routine measurement of insulin sensitivity or the dose of glucose to two hours post- load, but simply includes the determination of fasting blood glucose (NCEP, 2001). This definition of metabolic syndrome requires having at least 3 of the following 5 factors:

-increased waist circumference (≥ 102 cm in men and ≥88 cm in women),

-hypertriglyceridemia (≥150 mg/dl),

-low HDL cholesterol (<40 mg/dl in men and <50 mg/dl in women),

-hypertension (≥130/85 mmHg or treatment for hypertension)

-fasting glucose ≥110 mg/dl (or ≥100 mg/dl, as suggested by the panel of experts American Diabetes Association in 2003).

In addition, ATP III recommends a number of optional measures, such as C-reactive protein as a marker of proinflammatory state, and fibrinogen, as a marker of a prothrombotic state. However, the 5 criteria and cut-off values and the proposed by NCEP -ATP III panel have some problems as well as the criteria of the WHO. These criteria represent a consensus of experts and do not reflect a process of evidence-based field data, ie observations of epidemiological and take into account the influence for example of the breed and the age even more, the individual components of the metabolic syndrome. In addition, it is possible that the current criteria undergo changes when there will be more evidence on the role of fibrinogen or C-reactive protein, which could then be included as diagnostic criteria. In general, studies performed using the definitions proposed by WHO and ATP III show that they identify individuals with metabolic syndrome with good overlap (Ford ES). However, in 2009 there was an update of ATPIII in which fasting glucose levels and waist circumference were lower: fasting glucose ≥100 mg/dl and waist circumference ≥ 94 cm in men and ≥80 cm in women (Alberti KG).

Insulin resistance

The first definition of insulin resistance (IR) was formulated in 1922 by Banting and Best, the two researchers that identified insulin, according to which it was to be considered an insulin-resistant subject that it needed to produce more than 200 units of insulin per day to maintain an acceptable metabolic control (Rosenfeld L). Only with the acquisition of the measurement techniques of insulin, the IR has acquired a pathophysiological characterized more accurately. Berson and co-workers first, in the 70s, began to indicate hyperinsulinemia as marker of a condition in which they are required higher concentrations of insulin for evoke a physiological response (Yalow RS). The concept of IR was further expanded as a result definition of molecular biological and pathophysiological actions of insulin. Eventually '70s, Kahn defined the IR as any condition in which physiological insulin concentrations produce a biological response lower than normal (Kahn CR). Insulin resistance may involve several organs and tissues (liver, skeletal muscle and adipose tissue) or a single cell type (for example, the skeletal muscle cell) (Wyne KL; Roden M).

The gold standard for defining insulin resistance is the euglycemic hyperinsulinemic clamp technique. The hyperinsulinemic euglycemic clamp is the reference method for measuring tissue sensitivity to exogenous insulin. This complex test is based on amount of insulin infusion, for 120 minutes, such as to bring blood levels of the hormone, at a constant level of 100 microU/ml. Measuring the amount of glucose required to maintain normal blood glucose levels evaluates the ability of glucose uptake in the tissues by exogenous insulin. Since this test is difficult to apply on epidemiological studies and very expensive, there are other methods of assessment of insulin resistance such as Homoeostasis Model Assessment of Insulin Resistance (HOMA-IR), an index based on a mathematical model that considers the steady-state serum concentrations of fasting glucose and insulin, calculated as: HOMA index = (glucose mmol/L*insulin mU/L)/22.5. (Matthews DR)

In a single individual the phenomenon may extend to many biological processes, such as the glucose regulation, lipid and protein metabolism, or to be limited to a few hormonal actions, such as regulation of glycogen synthesis and/or glucose oxidation.

Glucose metabolism is the cornerstone of the metabolic actions of insulin, then the IR was related to reduced ability of insulin to promote the glucose uptake and a reduced lipolysis suppression. The resulting increase in free fatty acids, through a defined competition substrate mechanism, even more inhibits the utilization of glucose. This creates a vicious cycle that is able to worsen insulin resistance (the Randle cycle) (Randle PJ). According to studies carried out it has been developed by Reaven a further definition of IR as a reduced ability to induce consumption of glucose in skeletal muscle (44 Reaven GM, 2000). The skeletal muscle, in fact, is responsible for 70 % of the consumption of glucose in the post- absorptive. Later it was proposed additional definitions of IR. In 1991, Flier defined IR as a state in which a given concentration of insulin is associated with a subnormal response of glucose metabolism (Moller DE). In physiological conditions, insulin stimulates the glucose uptake into insulin-dependent target tissues, binding to its receptor on the cell membrane. This binding promotes phosphorylation of three tyrosine residues of the insulin receptor substrate (IRS-1) located in the cytoplasm. The phosphorylated peptide converts the

glicerofosfolipide phosphatidylinositol-4,5-bisphosphate (PIP2) in phosphatidylinositol -3,4,5-triphosphate (PIP3) by the enzyme phosphatidylinositol-3-kinase (PI3K), which activates in turn the Akt insulin-sensitive protein, also known as protein kinase B. The activation of Akt promotes, in metabolic tissues, the translocation of the glucose transporter (GLUT 4) to the membrane with consequent intracellular glucose uptake, while in endothelial cells the activation of this pathway results in phosphorylation of endothelial nitric oxide synthase (eNOS). This enzyme catalyzes the production of nitric oxide (NO) which plays a key role in endothelial vasodilation. Therefore, insulin resistance is often associated with endothelial dysfunction with impaired production/activation of eNOS and NO.

Metabolic syndrome and cardiovascular risk

Metabolic syndrome affects one in five people, and prevalence increases with age. Some studies estimate the prevalence in the USA to be nearly 25% of the population (Park YW). Subjects with metabolic syndrome have high risk to develop type 2 diabetes mellitus and have greater risk of mortality for cardiovascular disease (Lakka HM). Several epidemiological studies have identified an increased cardiovascular disease risk in individuals with MS (Isomaa B). Insulin resistance, metabolic disorders and vascular alterations are often present in individuals affected by visceral obesity: this condition, therefore, gives to these individuals an high cardiovascular risk (Lebovitz HE). Metabolic syndrome is gradually asserting, among the factors that can promote atherosclerosis, as one of the leading causes for cardiovascular events, the primary cause of death in industrialized countries. Cardiovascular diseases are among the chronic degenerative diseases with the highest morbidity and a leading cause of disability.

Recent epidemiological studies have shown the reversibility of risk, ie the possibility of reducing or delaying the onset of events through the reduction of risk factors. Mortality and disability that can cause these diseases are the reasons why people devote a large space for public health to prevent them.

1.2 ENDOTHELIAL PROGENITOR CELLS

Definition

The endothelial progenitor cells (EPCs) are a population of circulating cells, derived from bone marrow, functionally and phenotypically distinct from adult endothelial cells, with clonal expansion ability (ability of a single cell to multiply), proliferative and differentiative capacity. They play an important pathophysiological role: in fact they increase angiogenesis through the secretion of growth factors, constitute a rich source of circulating progenitor cells, are involved in the processes of tissue ischemia, in atherosclerosis, in endothelial dysfunction and in the tumor vasculature.
In 1997, Asahara and collaborators identified for the first time a population of circulating cells with properties similar to those of embryonic angioblasts, however able to differentiate *ex vivo* into an endothelial phenotype. These cells were defined Endothelial Progenitor Cells (Asahara T).

The importance of this discovery lies in exceeding the paradigm that postnatal neovascularization is mediated exclusively by the proliferation and remodeling of mature endothelial cells from pre-existing vessels. In fact, the work of Asahara et al has confirmed the crucial role of EPCs in the maintenance of endothelial function of blood vessels through a continuous process of re-endothelialization and neovascularization (Heiss C). This process consists of several stages characterized by the production of specific molecular factors: mobilization of EPCs from bone marrow, adhesion and transmigration in the endothelium, chemotaxis - migration - invasion and differentiation into mature endothelial cells. The failure/dysfunction of one or more of these stages causes a decreased or absent activity of EPCs. However, the normal range for the number of circulating EPC in healthy subjects, has not yet been defined.

There are two different approaches for the identification and characterization of EPCs: the flow cytometric technique and the cell cultures.

The flow cytometric technique is based on the identification of EPCs through the evaluation of the surface antigen expression: stem cell and mature endothelial differentiation markers. This method has the advantage of being able to select a more homogeneous population of cells and to be able to identify multiple cell subsets simultaneously, but it is, however, complicated by the absence of unique and restrictive markers to the alleged EPCs, given the co-expression of surface antigens than those

expressed on the circulating hematopoietic cells and endothelial cells. Another important restriction of the flow cytometric method is the lack of information of the functional type. The *ex vivo* approach (cell cultures), in contrast, not only allows the identification and the evaluation of EPC number, but also provides information of the functional type. This method is based on the EPC isolation from the circulating mononuclear cells from peripheral blood and the assessment of their proliferative capacity (ability to form colonies, CFU), and differentiative ability into mature endothelial cells.

Origin and differentiation of EPCs

EPCs derived from the bone marrow hematopoietic stem cells (HSCs) and therefore express specific surface markers characteristic of stem cell such as CD133 and CD34. In the process of differentiation EPCs acquire early endothelial markers such as vascular endothelial growth factor receptor-2 (VEGFR-2), also known as kinase insert domain receptor (KDR) Progressively there is a loss of CD133, earlier stem cell marker, which leads to a subpopulation of EPCs with the phenotype CD34/KDR.

There are different phenotypes (CD34/CD133/KDR, CD133/KDR and CD34/KDR) that identify the population of EPCs, they have short half-lives, so they are transient and detectable even at the same time in the bloodstream.

The differentiation process gradually leads to the outline Circulating Endothelial Cells (CECs) characterized by the expression of mature endothelial markers such as CD31 (or PECAM-1, platelet cell adhesion molecule-1), VE- cad (vascular endothelial cadherin) and the vonWillebrand factor (vWF) accompanied by the loss of stem cell markers. In the processes of differentiation of CECs in mature endothelial cells (ECs), we are witnessing a high expression of all the specific markers of endothelial cells which line: VE -cadherin, vWF, endothelial nitric oxide synthase, CD146 and E - selectin.

Insulin resistance and vascular repair

Insulin resistance is a metabolic disorder which results in the impaired glucose homoeostasis, encountered in several conditions, including obesity, pre-diabetes and Type 2 diabetes mellitus. Insulin resistance is associated with impaired downstream

signal transduction when insulin binds to its receptor, so reducing glucose uptake in metabolic tissues. However, the metabolic effects of these molecular abnormalities have wider implications than disordered blood glucose regulation alone: dyslipidaemia, inflammation and a pro-thrombotic tendency are also hallmarks of insulin-resistant states (Hsueh W). The combined effect of these factors in insulin-resistant subjects results in a significantly increased risk of cardiovascular events (Booth GL, Cubbon RM, Grundy SM).

Although many questions remain regarding the natural history of atherosclerosis, it is commonly accepted that dysfunction of the vascular endothelium represents the earliest manifestation of the disease (Ross R). A closely coupled relationship between insulin resistance and endothelial dysfunction is now supported by a wealth of observational and mechanistic studies. In health, the damaging effects incurred through exposure to risk factors are mitigated by endogenous processes which regenerate damaged endothelium and preserve the structural and functional integrity of the endothelial monolayer (Dimmeler S). Mature endothelial cells have a finite proliferative capacity and have limited potential for repair. The discovery of EPCs by Asahara et al. stimulated intense interest in the role of these cells both in terms of new vessel formation and in the regeneration of damaged endothelium. Mobilization of EPCs from the bone marrow into the circulation occurs in response to growth factors and cytokines, including VEGF (vascular endothelial growth factor) and SDF-1α (stromal-cell-derived factor-1α), (Aicher A). Both VEGF and SDF-1α up-regulate bone marrow MMP-9 (matrix metalloproteinase-9) activity.

The correct process of EPC mobilization/proliferation comprises a cascade of different signals, the key role is played by the PI3K\Akt pathway as the phosphorylation of Akt leads to activation of eNOS in the stromal cells of the bone marrow resulting in the production of nitric oxide. It promotes the transformation of MMP-9 from the inactive to an active form; causing the detachment of the SCF (stem cell factor, also known as Kit-L, tyrosine-kinase receptor) from the membrane (mKit-L) to form the soluble SCF (sKit-L). It acts at the niche of the bone marrow where there are the endothelial progenitor cells and promotes their mobilization and diffusion in the blood vessels.

After mobilization into the circulation, EPCs home or migrate toward regions of endothelial injury, where they adhere and proliferate facilitating vascular repair. Chemokine signalling plays a major role in directing circulating progenitor cells to sites of injury. Up-regulation of SDF-1α in ischemic tissues is driven largely by tissue hypoxia (Ceradini DJ). Interaction between SDF-1α and CXCR4 (CXC chemokine receptor 4) then facilitates homing of EPCs to sites of injury. EPCs contribute to endothelial repair by two principal mechanisms: by proliferating to form new endothelial cells, or by releasing an array of pro-angiogenic cytokines and growth factors which stimulate the proliferation of other EPCs or local mature endothelial cells (Hur J; Sieveking DP).

Disorders of glucose regulation are associated with abnormalities in EPC biology, including reduced circulating numbers of EPCs, defective mobilization from bone marrow and impaired functional properties of EPCs implicit to their capacity to mediate endothelial repair. Flow cytometric and cell culture analyses demonstrate consistently fewer circulating EPCs across the spectrum of insulin-resistant states. Individuals with Type 2 diabetes have reduced levels of circulating EPCs, which are correlated with disease severity (Tepper OM, Egan CG; Fadini GP, 2005; Kusuyama T). Hyperglycaemia may partially explain this association, as Fadini et al. (Fadini GP, 2007) demonstrated reduced numbers of EPCs in individuals with impaired glucose tolerance compared with those with normal glucose regulation. EPCs were negatively correlated with glucose levels after a glucose challenge. EPCs were, however, negatively correlated with components of the metabolic syndrome and with HOMA-IR, in a separate study of subjects across a wide range of cardiovascular risk (Fadini GP, 2006). EPCs were also found to be lower in another study of obese men with the metabolic syndrome compared with non-obese healthy controls (Westerweel PE). In that study, EPCs were associated with BMI (body mass index) and correlated inversely with components of the metabolic syndrome, but insulin resistance was not studied separately. Reduced circulating EPCs may be attributable to a number of factors, including defective mobilization, decreased proliferation and shortened survival in the circulation (Aicher A, 2003; Aicher A, 2004; Hristov M). As discussed in further detail

below, insulin resistance is closely associated with abnormalities in NO bioavailability and PI3K/Akt signalling, both of which play a crucial role in EPC mobilization from the bone marrow (Aicher A, 2003; Hristov M; Dimmeler S; Thum T; Werner C; Urao N). EPCs from humans and animals with Type 2 diabetes have multiple functional defects *in vitro*, including impaired migration to chemotactic stimuli, reduced proliferative potential and diminished ability to form vascular-like structures, which are likely to limit their regenerative capacity (Tepper OM; Ii M). These functional deficits appear to be biologically relevant *in vivo*, as Ii et al. (Ii M) have demonstrated that re-endothelialization following endothelium-denuding injury was impaired in mice with Type 2 diabetes (Ii M). Unfortunately, such studies do not allow the effects of insulin resistance to be considered separately from those of hyperglycaemia and other metabolic derangements characteristic of diabetes. Homing of EPCs to sites of vascular injury is dependent on an interaction between locally produced chemokines and the CXCR4 receptor on EPCs. Diabetes is associated with a decreased expression of the chemokine SDF-1α in injured tissues (Badillo AT) and reduced expression of CXCR4 in peripheral mononuclear cells (Egan CG), which may inhibit recruitment of EPCs from the circulation.

1.3 ENDOTHELIAL FUNCTION

Endothelium

The endothelium is a ubiquitous organ responsible for the regulation of hemodynamic and metabolic processes and it is also involved in the synthesis and inflammatory processes. The endothelial cells occupy an important position between the blood and the tissues; this location facilitates their involvement in numerous physiological processes; these cells are very thin and closely linked to each other, so that the endothelial surface does not present any discontinuity (except for sinusoids); typically, they assume an elongated shape towards the direction of blood flow, especially in the arterial vessels of greater caliber; in the capillaries, they are characterized by the extreme thinness. The endothelium is not a simple inner lining of the vessels but it is considered a real organ composed by one trillion cells that together weigh as the liver. The endothelium can be considered an autocrine and paracrine

organ able to secrete, in response to a large variety of signals, numerous chemical mediators that influence either the same cells that produced them that those nearby. The result is a modulation of vascular tone and blood flow in response to nervous, humoral and mechanical stimuli. The endothelial functions are several and very complex (and even more numerous are the mediators produced by its cells):

• Barrier function: the endothelium is similar to a semi-permeable membrane that controls the passage of substances from the extracellular fluid to bloodstream and vice versa;

• Regulation of coagulation, fibrinolysis and platelet aggregation; blood fluidity balance

• Control of leukocyte adhesion and infiltration

• Control of the proliferation of smooth muscle cells of the tunica media; modulation of tone, vascular permeability and structure; it plays a major role in the remodeling observed in hypertension, in re-stenosis after percutaneous coronary intervention and in the atherosclerosis

• Formation of new blood vessels (angiogenesis)

• Oxidation of LDL and regulation of inflammatory processes

The chemical mediators produced by the endothelium can be distinguished in vasodilators, which increase the lumen of the vessels with anti-proliferative, anti-thrombotic and anti-atherogenic action, and vasoconstrictors, which instead have opposite function.

Nitric oxide (NO) is the most important mediator of normal endothelial function: it is a potent vasodilator and it inhibits platelet activation, smooth muscle cell migration and proliferation, and leukocyte adhesion and activation. Consequently, the altered nitric oxide production has been associated with vascular diseases such as atherosclerosis, diabetes or hyperlipidemia. Endothelial dysfunction is characterized by an irregular secretion of nitric oxide and a resistance in states of abnormal insulin resistance.

Nitric Oxide

The nitric oxide is a bioactive product produced by several types of mammalian cells, with the lowest molecular weight among all cellular mediators known (Nathan C).

It is an intra and extracellular mediator virtually ubiquitous involved in several pathophysiological processes such as neurotransmission, inflammatory response and immune vascular homeostasis. It is produced by many cell types, including endothelial cells, monocytes, macrophages and neurons. The predominant effect of nitric oxide depends on the site production, the magnitude of its output and the type of target tissue. The biological half-life is a function of oxygen tension and the superoxide anion concentration and caries directly with the gas concentration in the solution. We consider that a NO concentration of 10-50 nm has a half-life of 3-5 sec, while a concentration of 300 nm has a half-life of 30 seconds (Ignarro LJ). Currently, nitric oxide is used as a mediator of numerous cell functions, including the following: (Sheferd JT; Stuehr DJ)

-vascular and intestinal smooth muscle, arterial and venous vasodilation, peristalsis inhibition
- platelet aggregation and adhesion inhibition
- cell proliferation inhibition
- immunomodulatory and cytotoxic effect of macrophages, polymorphonuclear cells, lymphocytes and other cells involved in immune defence
- neurotransmission
- mutagenicity

Mechanisms of action of Nitric Oxide

The vasodilation, antiaggregant and neuromodulatory actions of NO are due to the stimulation of a soluble guanatocyclase in the smooth muscle vascular cells, in platelets, neurons and other cells. The binding to guanylyl cyclase cause a change in its conformation with activation of catalytic site which follows an increase of ATPasi Na-K activity and an increase of cGMP synthesis from guanosintriphosphate. Two guanylyl cyclase isoforms are present, one cytosolic or soluble, activate by NO and the other one bind to the membrane, activate by atrial natriuretic peptide. The soluble

guanylyl cyclase is an eterodimeric protein of 150 kDa, containing a heme mole and one Cu^{++} for dimero mole (Ignarro LJ). The nitric oxide affinity constant for heme is 3000 times greater than oxygen. Paramagnetic NO binds with high affinity to the Fe^{++} heme hemoprotien, forming a nitrosil-heme adduct more stable than NO (Nathan C). On the other side, NO rapidly reacts with oxygen and free radicals derivates, as superoxide anion: this explains the protective effect of superoxidedesmutase on NO activity. NO binding the heme of soluble guanylyl cyclase, forms a ternary complex enzyme nitrosyl-heme, determining an increase of enzyme activity of 50-200 times.

Nitric oxide synthases

Nitric oxide is synthesized from guanidine nitrogen of L-arginine, which then gives rise to nitric oxide and L-citrulline. This reaction is catalyzed by a family of enzymes called NO synthase (NOS) (Forstermann U). Nitric oxide synthase can be present in plasma membrane, cytoplasm, nucleus, rough endoplasmic reticulum and mitochondria of cells. There are three isoforms of the NOS: nNOS (type 1 NOS), which was first discovered in neuronal tissues; iNOS (type 2 NOS), which was originally found to be inducible under certain conditions in macrophages and hepatocytes and eNOS (type 3 NOS), which was first identified in endothelial cells. These NOS isoforms are encoded by three different genes, and their nucleotide sequences are 51–57% homologous (Alderton WK). In general, eNOS, nNOS and iNOS are localized primarily in plasma membrane and cytoplasm, mitochondria and cytoplasm, and cytoplasm, respectively; nNOS and eNOS are expressed constitutively at low levels in a variety of cell types and tissues, whereas iNOS is normally not expressed at a significant level in cells or tissues (17 Wu G). When induced by certain immunological stimuli (including inflammatory cytokines or bacterial endotoxin), iNOS is highly expressed in many cell types and produces a large amount of NO. Due to its high affinity for calcium and tight binding with calmodulin, iNOS is fully active in the absence of exogenous Ca^{2+} or calmodulin. All of the NOS isoforms can be induced under certain stimuli through transcriptional and translational mechanisms (Wu G, 1998). Structurally, all NOS isozymes are homodimers. In functional NOS, the C-terminal reductase domain of one monomer (with binding sites for NADPH, FMN, and FAD) is linked to the N_terminal

oxygenase domain of the opposite monomer. This oxygenase domain carries a prosthetic heme group. The oxygenase domain also binds the tetrahydrobiopterin (BH4), molecular oxygen, and the substrate L-arginine. In a first step, NOS hydroxylates L-arginine to N^G-hydroxy-L-arginine. In a second step, NOS oxidizes N^G-hydroxy-L-arginine to citrulline and NO, (image 1) (Griffith OW).

Image 1: Schematic representation of the NO synthesis reaction (Daff S Nitric Oxide 23,2010)

Compelling evidence shows that NO synthesis is regulated not only by the amount and/or phosphorylation of the NOS protein, but also by the availability of cofactors (particularly NADPH, BH4 and Ca^{2+}) and Arginine (image 2). (Wu G, 2002). Although the Km values of NOS for Arginine are 3 to 20 μM, depending on its isoform, increasing extracellular Arginine concentrations from 0.05 to 5 mM dose dependently increases NO synthesis in a variety of cells, including activated macrophages and endothelial cells (Wu G, 1998). It is clear that Arginine increases the transcription of iNOS in macrophages and BH4 synthesis in endothelial cells (Shi W) whereby Arginine stimulates NO production in both cell types.

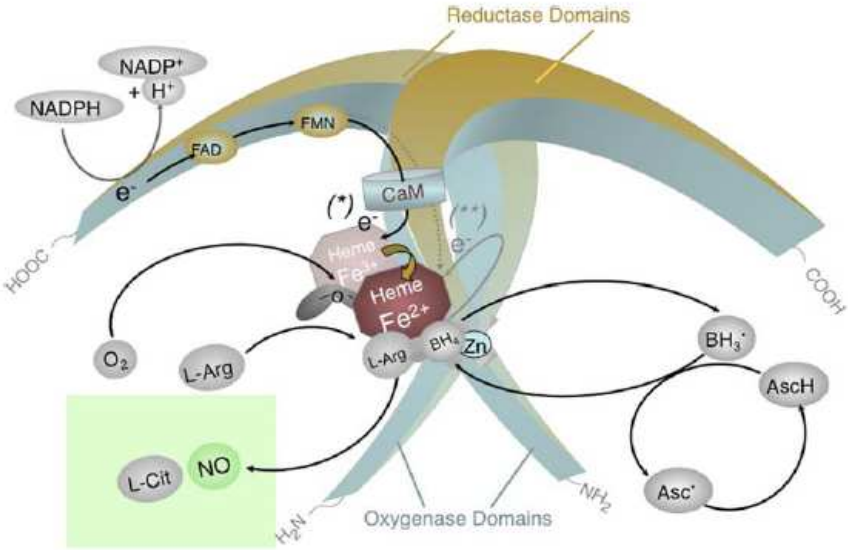

Image 2: endothelial nitric oxide synthase with ist substrate and cofactors. (Forstermann U Eur J Physiol 459, 2010)

L-arginine

L-arginine (2-amino-5-guanidino-pentanoic acid) is a conditionally essential, proteinogenic amino acid that is a natural constituent of dietary proteins. L-arginine is a basic amino acid (AA) in physiological fluids. Besides its role in protein metabolism, L-arginine is involved in various metabolic pathways, such as synthesis of creatine, L-ornithine, L-glutamate, and polyamines. Decarboxylation of L-arginine can produce agmatine, a biogenic amine metabolite. L-arginine is also involved in protein degradation by the ubiquitin-proteasome pathway. A biologically important pathway involves L-arginine as the substrate of a family of enzymes named nitric oxide synthases. The reaction mechanism of NO synthases involves a 2-electron transfer from molecular oxygen via a number of cofactors to L-arginine, resulting in the release of NO and L-citrulline (Wu G, 2009).

Its content is relatively high in seafood, watermelon juice, nuts, seeds, algae, meats, rice protein concentrate, and soy protein isolate (King DE), but low in the milk of most mammals (including cows, humans, and pigs) (Wu G, 1994). Results of the third

National Health and Nutrition Examination Survey indicate that mean Arginine intake for the US adult population is 4.4 g/day, with 25, 20 and 10% of people consuming<2.6 (suboptimal), 5–7.5, and>7.5 g/day, respectively (King DE).

Substantial amounts of orally administered Arginine do not enter the systemic circulation in adults, because 40% of dietary Arginine is degraded by the small intestine in first pass metabolism (Wu G, 2009). Although it is often stated that Arginine is formed in the mammalian liver, there is no net synthesis of Arginine via the hepatic urea cycle because the liver contains an exceedingly high Arginase activity to hydrolyze Arginine into urea plus ornithine (Wu G, 1998). Indeed, Arginine concentrations in hepatocytes are very low (0.03–0.1 mM), compared with 0.5–10 mM for other aminoacids (Li P). In adults, endogenous synthesis of Arginine involves the intestinal-renal axis (Wu G, 1998). Namely, citrulline is synthesized from glutamine, glutamate and proline in the mitochondria of enterocytes, released from the small intestine, and taken up primarily by kidneys for Arginine production. Interestingly, the uptake of citrulline by liver is negligible and this organ is not active in extracting Arginine from the circulation (Wu G, 2007). Therefore, nearly 100 and 90% of the gut-derived citrulline and Arginine, respectively, bypass the liver in pigs (Wu G, 2007). Similar patterns of citrulline and Arginine metabolism have recently been reported for humans (Van De Poll FW). Pyrroline-5-carboxylate (P5C) synthase, proline oxidase, and N-acetylglutamate (NAG) synthase are the three key regulatory enzymes of intestinal citrulline synthesis (Wu G, 1998). The enterocyte is the only mammalian cell type that expresses all three of these enzymes, indicating an essential role for the gut in whole-body homeostasis of citrulline and Arginine (Flynn E). In mammals, when dietary levels of Arginine are high, intestinal synthesis of citrulline from glutamine and glutamate may be inhibited for sparing of glutamine and glutamate for other metabolic pathways. Besides the kidney, citrulline is readily converted into Arginine in nearly all cell types, including adipocytes, endothelial cells, enterocytes, macrophages, neurons, and myocytes (Wu G, 1998).

Studies with macrophages and endothelial cells demonstrated that citrulline is transported into cells by the N system which is selective for aminoacids with a side-

chain amide group (e.g., glutamine and asparagine). Inside cells, conversion of citrulline into Arginine via Argininosuccinate synthase and lyase is the only pathway for citrulline utilization (Wu G, 1998).

Arginine transport by cells involves the system y^+ (a high-affinity, Na^+-independent transporter) and Na^+ dependent transporters (e.g., $b0^+$, $B0^+$, and y^+L) in a cell-specific manner (Grillo MA). The system y^+ is the principal high-affinity cationic amino acid transport (CAT) system expressed in NO-producing cells that transports arginine from the blood circulation into cells. Besides system y^+, systems y^+L, $b0^+$, and $B0^+$ have been characterized for transport of a wider range of substrates including cationic and neutral amino acids.

In endothelial cells, transport of L-arginine and other cationic amino acids is mediated principally via the Na^+-independent cationic amino acid transport systems y^+ and y^+ L, with negligible entry mediated by Na^+-dependent or Na^+-independent transport systems $B0^+$ or $b0^+$, respectively, or passive diffusion.

Within the y^+ system, 4 related CAT proteins are identified (CAT1 to CAT4), with 2 variants of CAT2, namely CAT2A and CAT2B. The CAT isoforms are the main transporters of L-arginine across the cell membrane. CAT-1, CAT-2A, and CAT-2B are located in the plasma membrane, with CAT-1 being expressed ubiquitously (except for adult liver hepatocytes), CAT-2A being predominantly expressed in the liver, and CAT-2B usually induced under inflammatory conditions in various cells. CAT-3 is specifically expressed in the brain, whereas CAT-4 is designated by a cDNA in human placenta. In a quiescent state, intracellular arginine transport is mostly through the CAT-1 transporter. Arginine uptake in activated macrophages is increased through induction of the CAT-2 transporter once inside cells; there are multiple pathways for Arginine degradation to produce NO, ornithine, urea, polyamines, proline, glutamate, creatine, and/or agmatine (Wu G, 1998). These pathways are initiated by Arginase, three isoform of NOS, Arginine:glycine amidinotransferase, and Arginine decarboxylase. Quantitatively, 1-2% of metabolized Arginine are utilized for polyamine synthesis and constitutive NO production, respectively, in mammalian cells (Li H, 2001). In mammals, the Arginase pathway is quantitatively most important for

Arginine catabolism. Type-I Arginase is expressed abundantly in hepatocytes (Morris SM) and to a limited extent, in extrahepatic cells, including enterocytes of post weaning mammals, endothelial cells, mammary epithelial cells, macrophages, and red blood cells (only in primates) (Li H, 2001). In contrast, type-II Arginase is widely expressed at relatively low levels in virtually all mitochondria-containing extrahepatic cells (including neuronal, renal, vascular, and muscle cells) and plays an important role in regulating the synthesis of NO, proline and polyamines (Li H, 2002). Large amounts of Arginine (2.3 g/day in a 70 kg man) are utilized for the production of creatine via the interorgan cooperation of kidneys, pancreas, liver and skeletal muscle.

There is a complex compartmentalization of Arginine degradation at cellular, tissue, and whole-body levels, and dietary Arginine supplementation may be a necessary strategy to maintain Arginine homeostasis for good health and body functions under many physiological and pathological conditions (Wu G, 2009). Arginine metabolism is regulated by multiple factors that include dietary components (e.g., lysine, manganese, n-3 fatty acids), hormones (e.g., glucocorticoids, growth hormone, and leptin), cytokines, endotoxins, and endogenously generated substances (e.g., creatine, lactate, ornithine, P5C, and methylarginines).

Lysine competes with Arginine for entry into cells and also inhibits Arginase activity (Wu G, 1998). Therefore, the dietary Arginine:lysine ratio is a critical factor influencing the effect of Arginine supplementation. Under normal feeding conditions, the total amount of Arginine in the diet should not be 150% greater than that of lysine (namely, Arginine/lysine<2.5). It is of interest to notice that these two dibasic aminoacids have a rate of entry into the brain proportional to its own concentration in the blood throughout a range of normal levels. However when the blood concentration of either of these aminoacids was raised above physiological levels the rate of entry ceased to be proportional to the blood concentration and increased less rapidly than at lower levels. In addition there is an inhibition of the entry of L-arginine into the brain if the concentration of l-lysine is raised to levels above the physiological and in the same way when L-arginine concentration rises up.

Modulation of endothelial cell L-arginine transport

L-arginine transport could be modulated by oxidatively modified low-density lipoproteins (LDL) and lysophosphatidylcholine (LPC). At physiological plasma concentrations, entry of L-arginine is mediated preferentially via a CAT system. Endothelium-derived NO synthesis and/or bioavailability is impaired in atherogenic vessels. Lysophosphatidylcholine and oxidized low-density lipoproteins (oxLDL) were proposed by Kikuta et al. (314) to inhibit NO production in endothelial cells by inhibiting a high-affinity transport system for L-arginine. Increasing extracellular L-arginine concentrations partially restores NO synthesis, with transport of L-arginine mediated preferentially via a low-affinity but high-capacity transport system(s). This model proposes that impaired eNOS activity can be restored by increased transport of L-arginine via a low-affinity carrier.

Role of caveolae

Caveolae are specialized invaginations of the plasma membrane (50–100 nm diameter) also known as "lipid rafts" formed as a result of localized accumulation of cholesterol, glycosphinogolipids, and structural proteins. The discovery of highly organized signalling molecules localized to membrane caveolae, including system y_ (CAT-1), eNOS, argininosuccinate synthase, receptors for insulin, provides an opportunity for investigating the role of caveolin-1 in modulating the L-arginine-NO signalling pathway in vascular endothelium.

It is possible to postulate a potential role of plasma membrane caveolae in modulating cationic amino transport in vascular endothelial cells. As shown before in endothelial cells uptake of L-arginine is mediated principally by systems y+ and y+L, and these transporters may be sequestered near or in plasmalemmal caveolae and that systems y+L and y+ mediate high-affinity transport of L-arginine. Co-localization of eNOs and the systems Y+ in caveolae in part explains the "arginine paradox" related to the phenomenon that in certain disease states eNOS requires an extracellular supply of L-arginine despite having sufficient intracellular L-arginine concentrations.

References

Aicher A, et al., 2003, Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat. Med. 9, 1370–1376

Aicher A, et al., 2005, Mobilizing endothelial progenitor cells. Hypertension 45, 321–325

Aicher, A et al., 2004, The role of NOS3 in stem cell mobilization. Trends Mol. Med. 10, 421–425

Alberti KG, et al., 2009, Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 20;120(16):1640-5.

Alderton WK, Cooper CE et al., 2001 Nitric oxide synthases: structure, function and inhibition Biochem J 357:593-615.

Asahara T et al., 1997, Isolation of putative progenitor endothelial cells for angiogenesis. Science

Avogaro P, et al., 1967, Association of hyperlipemia, diabetes mellitus and mild obesity. Acta Diabetol Lat 4:572-90.

Badillo AT, et al., 2007, Lentiviral gene transfer of SDF-1α to wounds improves diabetic wound healing. J. Surg. Res. 143, 35–42

Booth, GL, et al., 2006, Relation between age and cardiovascular disease in men and women with diabetes compared with non-diabetic people: a population-based retrospective cohort study. Lancet 368, 29–36

Ceradini DJ et al., 2004, Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat. Med. 10, 858–864

Cubbon, RM, et al., 2007, Temporal trends in mortality of patients with diabetes mellitus suffering acute myocardial infarction: a comparison of over 3000 patients between 1995 and 2003. Eur. Heart J. 28, 540–545 14;275(5302):964-7

De Fronzo RA, Ferrannini E, 1991, Insulino-resistenza. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 14:173-94.

Dimmeler S, et al., 2001, HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J. Clin. Invest. 108, 391–397

Dimmeler S and Zeiher AM, 2004, Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? J. Mol. Med. 82, 671–677

Egan CG, et al., 200,) Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes. Diabetologia 51, 1296–1305

Erkelens DW et al., 1993, Tulp syndrome. Lancet 18:1536-7.

Fadini GP, et al., 2005, Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J. Am. Coll. Cardiol. 45, 1449–1457

Fadini GP, et al., 2006, Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. Eur. Heart J. 27, 2247–2255

Fadini GP, et al., 2007 Glucose tolerance is negatively associated with circulating progenitor cell levels. Diabetologia 50, 2156–2163

Ferrannini E et al., 1991, Hyperinsulinaemia: the key feature of a cardiovascular and metabolic syndrome. Diabetologia 34:416-22.

Flier JS, Mntzoros C, 2001, Insulin resistance: definition and clinical spectrum.

Flynn NE, Wu G, 1996, An important role for endogenous synthesis of arginine in maintaining arginine homeostasis in neonatal pigs. Am J Physiol Regul Integr Comp Physiol 271:R1149–R1155.

Ford ES, Giles WH, 2003, A comparison of the prevalence of the metabolic syndrome using two proposed definitions. Diabetes Care 26:575-81.

Forstermann U, et al., 1993, Isoforms of nitric oxide synthase: functions in the cardiovascular system. Eur Heart J 14(Suppl 1):10-15.

Griffith OW, Stuehr DJ, 1995, Nitric oxide synthases – properties and catalytic mechanism. Annu Rev Physiol 57: 707-736.

Grillo MA, Lanza A, et al., 2008, Transport of amino acids through the placenta and their role. Amino Acids 34:517–523.

Grundy, SM, et al., 2001, Cardiovascular risk assessment based on US cohort studies: findings from a National Heart, Lung, and Blood institute workshop. Circulation 104, 491–496

Haller H, 1977, Epidemiology and associated risk factors of hyperlipoproteinemia. Z Gesamte Inn Med 32:124-8.

Heiss C et al., 2005, Impaired progenitor cell activity in age-related endothelial dysfunction. J Am Coll Cardiol. 3;45(9):1441-8.

Hristov M, et al., 2003, Endothelial progenitor cells: mobilization, differentiation, and homing. Arterioscler. Thromb. Vasc. Biol. 23, 1185–1189

Hsueh, W et al., 2004, Insulin resistance and the endothelium. Am. J. Med. 117, 109–117

Hur J, et al., 2004, Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler. Thromb. Vasc. Biol. 24, 288–293
Ignarro LJ,1990, Biosynthesis and metabolism of endothelium- derived nitric oxide Annu Rev Pharmacol Toxicol 30:535-560.

Ii M, et al., 2006, Endothelial progenitor thrombospondin-1 mediates diabetes-induced delay in reendothelialization following arterial injury. Circ. Res. 98, 697–704

Isomaa B et al., 2001, Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care 24: 683-9.

Kahn CR, 1978, Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. Metabolism 27(12 Suppl 2:1893-902

Kaplan NM, 1989, The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. Arch Intern Med 149:1514-20.

King DE, et al., 2008, Variation in L-arginine intake follow demographics and lifestyle factors that may impact cardiovascular disease risk. Nutr Res 28:21–24

Kusuyama T, et al., 2006, Effects of treatment for diabetes mellitus on circulating vascular progenitor cells. J. Pharmacol. Sci. 102, 96–102

Lakka HM, et al., 2002, The metabolic syndrome and total andcardiovascular disease mortality in middle aged men. JAMA 288: 2709-2716.

Lebovitz HE, Banerji MA, 2005, Point: visceral adiposity is causally related to insulin resistance. Diabetes Care 28(9): 2322–2325

Li H, et al., 2001, Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. Am J Physiol Endocrinol Metab 280:E75–E82

Li P, Kim SW, Li XL et al., 2008a, Dietary supplementation with cholesterol and docosahexaenoic acid increases the activity of the arginine-nitric oxide pathway in tissues of young pigs. Nitric Oxide 19:259–265.

Matthews DR, et al., 1985, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia Jul;28(7):412-9
45 Moller DE, Flier JS, 1991, Insulin resistance mechanisms, syndromes and implications. N Engl J Med. 325(13):938-48.

Morgagni GB, 1975, The seats and causes of diseases investigated by anatomy De sedibus et causis morborum per anatomen indagata. Vol. 1.

Morris SM Jr, 2007, Arginine metabolism: boundaries of our knowledge. J Nutr 137:1602S–1609S.

Nathan C, 1992, Nitric oxide as a secretory product of mammalian cells. FASEB J 6:3051-3064.

Shepherd JT, Z. Katusic, 1999, Endothelium-derived vasoactive factors: I. Endothelium-dependent relaxation. Hypertension 18: III-76- III-85.

Park YW, et al.,2003, The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988–1994. Arch Intern Med 163: 427–36.
Randle PJ, et al., 1963, The glucose fatty-acid cycle. Its role in insulin sensivity and the metabolic disturbances of diabetes mellitus. Lancet 13;1(7285):785-9.

Reaven GM,1988, Role of insulin-resistance in human disease. Banting lecture. Diabetes 37:1595-607

Reaven GM, 2000, Insulin resistance and its consequences: type 2 diabetes mellitus and coronary heart disease. In Diabetes Mellitus: A Fundamental and Clinical Text., p.604 -615

Roden M et al., 1996, Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest 97:2859–65.

Rosenfeld L, 2002, Insulin: discovery and controversy. Clin Chem. 48(12):2270-88.

Ross R, 1999, Atherosclerosis: an inflammatory disease. N. Engl. J. Med. 340, 115–126

Shi W, Meininger CJ, et al., 2004, Regulation of tetrahydrobiopterin synthesis and bioavailability in ehdothelial cells. Cell Biochem Biophys 41:415-433.

Sieveking DP, et al., 200,) strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J. Am. Coll. Cardiol. 51, 660–668

Stuehr DJ, and Griffith OW, 1992, Mammalian nitric oxide synthases. Advances in Enzymology 65: 287-346.

Tepper OM, et al., 2002, Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 106, 2781–2786

Thum T, et al., 2007, Age-dependent impairment of endothelial progenitor cells is corrected by growth-hormone-mediated increase of insulin-like growth-factor-1. Circ. Res. 100, 434–443

Urao N, et al., 2006, Erythropoietin-mobilized endothelial progenitors enhance reendothelialization via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia. Circ. Res. 98, 1405–1413

Van de Poll MCG, et al., 2007, Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. Am J Clin Nutr 85:167–172
Yalow RS, Berson SA, 1970, Dynamics of insulin secretion in early diabetes in humans. Adv Metab
Disord. 1:Suppl 1:95

Werner C, et al., 2007, The peroxisome proliferator-activated receptor-γ agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance. Diabetes 56, 2609–2615

Westerweel PE, et al., 2008, Endothelial progenitor cell levels in obese men with the metabolic syndrome and the effect of simvastatin onotherapy vs. simvastatin/ezetimibe combination therapy. Eur. Heart J. 29, 2808–2817

Wyne KL, 2003, Free fatty acids and type 2 diabetes mellitus. Am J Med 115 (Suppl 8A):29S-36S.

Wu G, Knabe DA, 1994, Free and protein-bound amino acids in sow's colostrum and milk. J Nutr 124:2437–2444.

Wu G, Morris Jr SM, 1998, Arginine metabolism: nitric oxide and beyond. Biochem J 336:1-17.

Wu G, Meininger CJ, 2002, Regulation of nitric oxide synthesis by dietary factors Annu Rev Nutr 22:61-86.

Wu G, Bazer FW, Davis TA et al., 2007, Important roles for the arginine family of amino acids in swine nutrition and production. Livest Sci 112:8–22.

Wu G, et al., 2009, Arginine metabolism and nutrition in growth, health and disease Amino Acids 37:153-168.

2. STATE OF THE ART

Recently we have published a study to determine whether sucrose-induced insulin resistance could increase the expression of cardiac matrix metalloproteinases (MMPs), indices of matrix remodelling, and whether the addition of 1.25 g day (-1) of L-arginine (ARG) to a sucrose diet could prevent both the sucrose-induced metabolic abnormalities and elevated cardiac expression of matrix metalloproteinases in an insulin resistant stage that precedes frank type 2 diabetes. 38 male Sprague-Dawley rats were involved, 16 rats maintained a standard chow diet (ST), 12 rats were switched to a sucrose enriched diet (SU) and 10 rats to a sucrose plus L-arginine (1.25 g day (-1)) enriched diet (SU + ARG) for a period of 8 weeks. After 8 weeks of different diets, an intravenous glucose tolerance test (IVGTT) was performed. At the end of the study, retroperitoneal fat, heart weight/body weight ratio, fasting plasma glucose, serum insulin, and serum triglyceride levels and integrated insulin area after IVGTT were

significantly higher in SU than in SU + ARG and ST. All these parameters were comparable between SU + ARG and ST animals. FFA levels were significantly different among groups, with highest levels in SU and lowest levels in ST. Fasting plasma c-GMP levels and the integrated c-GMP area after IVGTT, an index of nitric oxide activity, were significantly lower in SU than in SU + ARG and ST, the result was similar in SU + ARG and in ST; MMP-9 protein expression increased 10.5-fold, MMP-2 protein expression increased 2.4-fold and the expression of tissue inhibitors of metalloproteinase (TIMP-1) increased 1.7-fold in SU rats as compared to ST animals. This was accompanied with a significant increase of cardiac triglyceride concentrations. SU rats developed insulin resistance and hyperlipidaemia, accompanied with increased fat deposition in the heart and enhanced MMP protein expression. Conversely, ARG supplementation prevents these metabolic abnormalities and restored MMP/TIMP-1 balance (Monti LD, 2008).

In humans, the intravenous infusion 0.5 g/min of L-arginine but not D-Arginine increased whole body glucose disposal, and blood flow in normal subjects (Paolisso G). McConnell et al. performed similar amount of L-arginine infusion in endurance trained males during exercise test (McConell GK). They found that L-arginine determined a significant increase of the whole body glucose disposal, a decrease of free fatty acid levels while insulin levels remained unchanged as compared to saline control study. These data strongly suggest that L-arginine infusion improved muscle glucose utilization modulating free fatty acid concentration during exercise. Insulin sensitivity and insulin-mediated vasodilation were also improved by infusing lower doses of L-arginine (0.52 mg $kg^{-1}min^{-1}$) in healthy, obese and type 2 diabetic subjects (Washer TC).

These effects were confirmed by a double-blind chronic treatment of L-arginine (9 g daily) in non obese type 2 diabetic patients. After one month of L-arginine therapy peripheral and hepatic insulin sensitivity were significantly improved and cyclic-GMP levels and insulin-mediated vasodilation were normalized (Piatti PM). Recently our group has published a clinical trial regarding the specific effect of L-arginine on adiposity in humans. This was a 21-day randomized, placebo-controlled trial in 33

hospitalized middle-aged, obese (mean BMI = 39.1±0.5 kg/m^2) subjects with diet-controlled Type 2 diabetes mellitus. During the study period, each patient received a low-caloric diet (1000 kcal/day) and a regular exercise-training program (4 min twice a day for 5 days/week). They were randomized to 8.3 g arg/day (approximately 80 mg/kg body weight per day) or placebo. As expected from the low caloric diet, both groups of subjects exhibited reductions in body weight, fat mass, waist circumference, circulating levels of glucose, fructosamine and insulin. Moreover, increase in antioxidant capacity and circulating levels of adiponectin were observed for these patients. Importantly all improvements were significantly greater in arginine group than in the placebo group. Additionally fat free mass was maintained in the L-arginine group but reduced by 1.6 kg in the placebo group. Thus, Arginine supplementation to obese subjects promoted fat reduction and spared lean body mass during weight loss (Lucotti P, 2006).

We evaluated also the effects of long-term oral L-arginine treatment on endothelial dysfunction, inflammation, adipokine levels, glucose tolerance, and insulin sensitivity in no diabetic patients with stable cardiovascular disease (coronary artery disease). Sixty-four patients with cardiovascular disease previously submitted to an aortocoronary bypass and not known for type 2 diabetes mellitus had an oral glucose load to define their glucose tolerance. Thirty-two patients with non diabetic response were eligible to receive, in a double-blind randomized parallel order, L-arginine (6.4 g/d) or placebo for 6 months. An evaluation of insulin sensitivity index during the oral glucose load, markers of systemic nitric oxide bioavailability and inflammation, and blood flow was performed before and at the end of the treatment in both groups. Compared with placebo, L-arginine decreased asymmetric dimethylarginine levels ($p<0.01$), and increased cyclic guanosine monophosphate ($p<0.01$), L-arginine to asymmetric dimethylarginine ratio ($p < 0.0001$), and reactive hyperemia ($p<0.05$). Finally, L-arginine increased insulin sensitivity index ($p <0.05$) and adiponectin ($p<0.01$) and decreased interleukin-6 and monocyte chemoattractant protein-1 levels. In conclusion L-arginine seems to have anti-inflammatory and metabolic advantages in these patients (Lucotti P, 2009). An anabolic effect of L-arginine on muscle gain is

achieved independent of changes in serum concentrations of insulin or growth hormone. Dietary arginine supplementation enhances insulin sensitivity and amplifies its signalling mechanisms on protein synthesis as well as the metabolism of glucose and fatty acids. So Arginine supplementation regulates the repartitioning of dietary energy to favour muscle over fat gain in the body. Recently Monti LD et al. performed a mono-centre, randomized, double-blind, parallel-group, placebo-controlled, phase III trial (named l-arg trial). In this study, 144 individuals, affected by impaired glucose tolerance (IGT) and metabolic syndrome (MS), received 6.4g/day of L-arginine, or placebo for 18 months plus a 12-month extended follow-up period after study drug termination, in order to prevent or delay type 2 diabetes and to normalize glucose tolerance in individuals at high risk for type 2 diabetes. The results showed that the supplementation of L-arginine for 18 months does not significantly reduce the incidence of diabetes but does significantly increase regression to normal glucose tolerance (NGT). (Monti LD, 2012).

In light of these data obtained with L-arginine oral supplementation, we considered a new way of administration of this amino acid, that could be more acceptable by patients, through an innovative food product containing L-arginine (at least 10%) with a low content of carbohydrates.

References

Lucotti P, et al., 2006, Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab 291:E906-912

Lucotti P, et al., 2009, Oral L-arginine supplementation improbe endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism 58: 1270-1276.

McConell GK, et al., 2006, L-arginine infusion increases glucose clearance during prolonged exercise in humans. Am J Physiol Endocrinol Metab 290:E60-E66.

Monti LD, et al., 2008, Beneficial role of L-arginine in cardiac matrix remodelling in insulin resistant rats. Eur J Clin Invest 38: 849-856

Monti LD et al., 2012, Effect of a long-term oral L-arginine supplementation on glucose metabolism: a randomized, double-blind,placebo-controlled trial. Diabetes, Obesity and Metabolism 14: 893–900.

Paolisso G, et al., 1997 L-arginine but not D-arginine stimulates insulin-mediated glucose uptake. Metabolism 46:1068-1073.

Piatti PM, et al., 2001, Long-term oral L-arginine administration improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients. Diabetes Care 24:875-880.

Washer TC, et al., 1997, Effects of low-dose L-arginine on insulin mediated vasodilation and insulin sensitivity. Eur J Clin Invest 27:690-695.

3. AIM

The use of L-arginine as oral food supplement to a normal diet, in relatively large doses, has been proved to have a salutary effect on cardiovascular diseases as extensively described in literature, both in animal and in humans studies. L-arginine was found to be bioavailable and effective in the prevention of impairment of glucose metabolism and endothelial dysfunction, improving endothelial-mediated blood flow.
In light of these data obtained with L-arginine oral supplementation, we formulated a new food product containing L-arginine, designed in the form of biscuit, as an alternative to a pharmacological approach; this product containing 10% of L-arginine and low levels of glycemic carbohydrates appears to be more palatable and acceptable by the subjects with IGT and metabolic syndrome. Previous data, obtained in a pilot postprandial study in healthy subjects, showed that in the biscuit enriched of L-arginine (6.6 gr in 6 biscuits), the L-arginine bio-availability is >99% and promotes a beneficial effect on endothelial and vascular function by increasing nitric oxide and its second messenger, cGMP. This PhD project is a double-blind crossover study performed in obese subjects with impaired glucose tolerance (IGT) and metabolic syndrome (MS), in order to evaluate the effects of the L-arginine-enriched biscuits consumption on endothelial function, glucose metabolism and on changes in body composition. Moreover we assessed *in vitro*, the effect of L-arginine on endothelial progenitor cells via Akt-eNOS pathway, both as gene expression and as protein expression of the two molecules. New peripheral markers of the beneficial effect of L-arginine on the modulation of endothelial progenitor cells were also evaluated.

4. RESULTS AND DISCUSSION

4.1 MATERIALS AND METHODS

4.1.1 Formulation of the biscuit

L-arginine-enriched biscuits were manufactured in a single batch by combining 20% L-arginine Kyowa (Eurosup, Castello D'Agogna, PV, Italy) with 26 % mixed cereal flakes (corn, oat, whole wheat), 18% puffed rice, 17% hazelnuts, and 17% candied orange peel. Biscuits were produced by mixing all ingredients with a minimum of water (about 4-8 % of total weight) to support homogenization. Then an aggregation/shaping process (patented) was performed by sonication of the mixture, using a prototype of sonotrode in titanium (20-40 kHz; Branson); the process was conducted at low temperature range, which prevents the degradation of the amino acid by Maillard reactions or other heat dependent modifications. A cylindrical mould was used to achieve the shape of the food product. The process was maintained for 20-2000 milliseconds at a starting temperature of 25-40 °C without temperature changes at the inner of the product till the end of the sonication process. To increase the shelf life of the food product, its water content was successively reduced by a batch drying process, at a temperature less than 60 °C in a static oven. The food product, ready to eat, was then conveniently packed and stored at room temperature. The resulting product was storage stable under normal conditions for an extended period of time. One single unit weight was about 10 g and comprises at least 1 g of L-arginine; as evaluated by appropriate analogic scale, the product had pleasant organoleptic properties and no undesirable after taste has been pointed out. Placebo biscuits without the addition of L-arginine were similarly formulated and obtained with the same technological process.

4.1.2 Subjects and study design

This 7-week study enrolled 15 obese subjects with IGT and MS (8 men/7 women, aged 62.5±3.5 years) in a randomized double-blind placebo-controlled crossover design. A baseline evaluation and oral glucose tolerance test was performed to recruit only patients with IGT and MS. The latter was defined according to ATP III, metabolic syndrome requires having at least 3 of the following 5 factors:

-increased waist circumference (≥ 102 cm in men and ≥88 cm in women),

-hypertriglyceridemia (≥150 mg/dl),

-low HDL cholesterol (<40 mg/dl in men and <50 mg/dl in women),

-hypertension (≥130/85 mmHg or treatment for hypertension),

-fasting glucose ≥110 mg/dl (or ≥100 mg/dl, as suggested by the panel of experts American Diabetes Association in 2003).

Namely in the presence of one or more risk factors for type 2 diabetes, including overweight (body-mass index [BMI] >25 kg/m², family history of type 2 diabetes (first degree relatives of patients with type 2 diabetes), and cardiovascular disease. Diagnosis of IGT was based on a fasting plasma glucose tests (FPGT) result of less than 7.0 mmol/L (less than 126 mg/dl) and a plasma glucose value of 7.8 mmol/L (140 mg/dl) or more, but less than 11.1 mmol/L (200 mg/dl) 2 h after the 75 g oral glucose load (OGTT). OGTTs were also repeated at the end of each intervention period.

Clinical and metabolic variables of involved subjects are reported in Table 1.

Age (years)	62.5 ± 3.5
Gender M:F	8 : 7
Weight (kg)	84.5 ± 4.2
BMI (kg/m2)	30.3 ± 1.5
Fat Mass (FFM, kg)	29.6 ± 3.0
Free Fat Mass (FM, kg)	62.5 ± 3.5
Waist (cm)	M: 108.8 ± 4.0/ F: 102.0 ± 4.3
Systolic Blood Pressure (mmHg)	121.3 ± 4.0
Diastolic Blood Pressure (mmHg)	76.0 ± 2.0
Fasting glucose levels (mg/dl)	113.2 ± 3.5
Fasting insulin levels (µU/ml)	9.4 ± 2.0
Total cholesterol levels (mg/dl)	160.0 ± 9.1
HDL cholesterol levels (mg/dl)	43.4 ± 3.1
Trigliceride levels (mg/dl)	96.3 ± 13.4
NOx (µmol/l)	17.9 ± 2.8
cGMP (pmol/mL)	7.4 ± 0.9
Basal forearm blood flow (ml/100 ml/min)	2.98± 0.24
Post-ischemic forearm blood flow (ml/100 ml/min)	5.73 ± 0.63

Table 1: Clinical and metabolic characteristics of the 15 participants of this study

Patients were randomized into two groups. One group consumed 6 biscuits containing a total amount of 6.6 g of L-arginine divided into two snacks (in the morning and in the afternoon) for 2 weeks, followed by the consumption of 6 placebo biscuits for 2 weeks, with a 2-week washout between the two study periods. The other group consumed these food preparations types in reverse sequence.

During each two-week intervention period the amount of energy derived from the biscuits (180kcal) was included in a 1600cal balanced hypo caloric diet containing 55% carbohydrate, 25%–30% fat and 15%–20% protein. During the washout period, a free diet was allowed. A complete 3-day food diary consisting of two working days and one holiday was kept three days before and during the last three days of the intervention periods to assess volunteer diet compliance. Food diaries were elaborated with dedicated software to decode foods (Nutritionist Pro 2.5, Axial System, Stafford, Texas), modified introducing the L-arginine contents, obtained from INRAN and USDA database, in about 800 different foods items. In table 2 is showed the composition of the diet in the subjects of the study.

Table 2

Composition of the diet in 15 volunteers affected by Obesity, Metabolic Syndrome and Impaired Glucose Tolerance during the treatment (L-arginine enriched biscuits) and control (placebo biscuit) periods of the study measured by a 3 day food diary (two working days and one holiday day).

	L-Arg enriched Biscuit	Placebo Biscuit	P value
kcal/d	1603±80	1606±78	0.89
Energy Intake (MJ/d)	6.70±0.3	6.72±0.3	0.89
Protein (% of energy)	16.4±4.6	18.8±3.7	0.18
L-arginine (g/d)	3.4±1.3	4.1±1.2	0.17
Carbohydrate (% of energy)	53.2±13.3	50.3±14.6	0.63
Fiber (g/d)	20.0±9.5	18.7±8.6	0.70
Fat (% of energy)	33.4 ±10.6	32.2 ±10.0	0.72
SFA (% of energy)	10.1±4.0	9.6±3.5	0.75
MUFA (% of energy)	11.4±5.8	12.4±7.0	0.68
PUFA (% of energy)	6.8±3.9	7.5±3.9	0.66
Cholesterol (mg/d)	255.8±47	232.3±80	0.67

All data are mean±SD. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Overnight fasting and during OGTT blood samples were collected at baseline and at the end of each intervention periods for serum/plasma biochemistries. In particular, samples for the evaluation of glucose, insulin, NOx and L-arginine levels were evaluated at 0, 30, 60, 90, and 120 minutes. c-GMP levels were evaluated at 0, 60, 90 and 120 minutes. Further, samples for the measurement of plasma glucose and serum insulin levels were drawn at 0, 30, 60, 90, and 120 minutes and fasting proinsulin, ADMA, MMP-9, VEGF-α, SCF (sKit-L), SDF-1α levels were also evaluated.

Basal blood pressure was taken in supine position after 10 min of rest, and the mean of two measurements was used as the value. Forearm blood flow (FBF) was measured by strain-gauge venous occlusion plethysmography. Before any measurement was taken, the hand circulation was occluded using a wrist cuff inflated to 240 mmHg. Baseline blood flow was calculated as the mean of at least three values. Reactive hyperaemia (endothelium-dependent vasodilation) was measured after the release of a 5-min arterial occlusion, produced by inflating a standard sphygmomanometer cuff on the upper arm to 100 mmHg above systolic blood pressure (SBP). These measurement were performed at basal and every 60 min until the end of the study. Body weight, fat mass and fat free mass distribution was evaluated by bioimpedenziometry using TANITA body fat analyzer (Tanita, Tokyo, Japan). The study design is reported in figure 1.

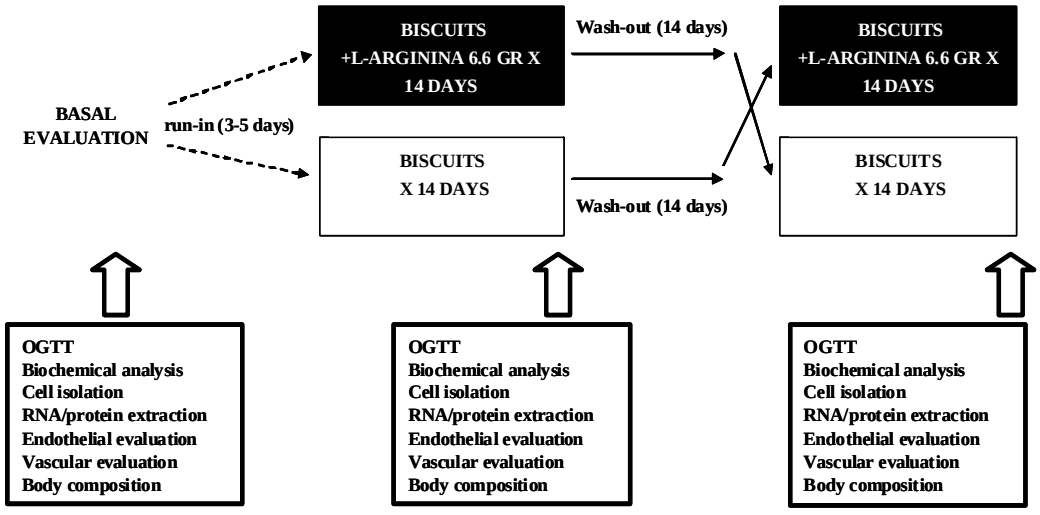

Figure 1: study design

4.1.3 Endothelial progenitor cell isolation and culture

Circulating EPCs were enumerated by flow cytometry as positive cell population to CD34 and KDR expression markers using a hierarchical gating strategy to count cells negative for CD45 marker or expressing only very low levels ($CD45^{dim}$). Briefly, 100 μL peripheral blood was incubated with the following monoclonal antibodies: PE-conjugated anti-human CD34 (BD, Franklin Lakes, NJ), allophycocyanin-conjugated anti-human KDR (R&D Systems, Minneapolis, MN), and PE-Cy7–conjugated anti-human CD45 (Beckman Coulter, Brea, CA). After incubation, 100 μL Flow-Count beads (Beckman Coulter) was added to the stained whole blood. To avoid the loss of cells and/or counting beads, a lyse-no-wash technique was used as follows: erythrocytes were lysed with ammonium chloride buffer (155 mmol/L ammonium chloride, 10 mmol/L potassium bicarbonate, and 0.1 mmol/L EDTA), and the sample was analyzed immediately on a FACSCanto II flow cytometer, equipped with FACSDiva software (BD Biosciences, San Jose, CA). The data analyses were processed with FCS Express (De Novo Software, Los Angeles, CA), and the number of $CD45^{dim}CD34^{+}KDR^{+}$ was expressed as number of cells per million of events.

The number of colony-forming-units of EPCs was measured as described by Hill et al. (Hill JM, 2003). Peripheral-blood mononuclear cells were isolated by Ficoll density-gradient centrifugation. Recovered cells were resuspended in growth medium (M 199 Sigma-Aldrich, St. Louis, MO USA), supplemented with 20% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin) and plated on endothelial cell attachment factor coated dishes (ECAF, Sigma-Aldrich, St. Louis, MO USA) After 48h, the no adherent cells were collected, sedimented by centrifugation, resuspended in culture medium and reseeded at 1×10^{6}/well in 24-well plates previously coated with endothelial cell attachment factor. Adherent cells were cultured for 7 days. At the end of culture, the number of colonies formed in 12 wells for each sample was counted.

4.1.4 Gene expression

Total RNA was isolated from the colonies formed on day 7 using the RNeasy Mini Kit (QIAGEN, Valencia, CA) with on-column DNase I digestion. Next, 2 μg total RNA

from each sample were reverse transcribed using superscript VILO cDNA synthesis kit (Invitrogen, Carisbad, CA, USA). Quantitative relative real-time PCR was used to measure the expression of eNOS and Akt genes. The PCR mix for SYBR Green assay was prepared using 10 uL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1 uL (1 pmol) sense primers, 1 uL (1 pmol) antisense primers, and 100 ng cDNA in a final reaction volume of 20 uL. The real-time PCR was performed in a Model 7900HT Fast Real-Time PCR System (Applied Biosystems Foster City, CA) under the universal cycling conditions consisting of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. At the end of the real time PCR a C_T value (cycle threshold) is obtain for each sample. The level of β-actin expression (housekeeping gene), was used to normalize the amount of cDNA added to each reaction to obtain ΔC_T value (difference between gene target C_T value and β-actin C_T value). Then the difference between ΔC_T of each sample and ΔC_T of the endogenous control ($\Delta\Delta C_T$) is used in the final formula $2^{-\Delta\Delta CT}$ to calculate gene expression levels.

4.1.5 Laboratory measurements

Glucose, total cholesterol, HDL-cholesterol and triglyceride levels were measured with spectrophotometric methods adapted to the Cobas MIRA using commercial kits (ABX, Rome, Italy)**.** Insulin, proinsulin, SCF (sKit-L), MMP-9, ADMA, SDF-1α and VEGF-α levels were assayed by ELISA commercial kits (Insulin ELISA, Mercodia, Uppsala, Sweden; Proinsulin ELISA, DRG, Marburg, Germany; MMP-9, Amersham Biosciences, Freiburg Germany; ADMA, Diagnostika GMBH Hamburg Germany, SDF-1α and SCF (sKit-L) R&D Systems Abingdon UK; VEGF-α, eBioscience, Vienna, Austria). NOx levels were evaluated by metabolic end product measurement; i.e., nitrite and nitrate, using enzymatic catalysis coupled with the Griess reaction. The c-GMP levels were measured by ELISA (Cyclic GMP, Assay Designs, Ann Arbor, MI, USA). L-arginine was extracted from plasma samples using cation-exchange Strata SCX 100-mg columns (Phenomenex) and assayed by high-performance liquid chromatography (Pi, J).

4.1.6 Chemical analysis

Biscuits' proximate composition was assessed by AOAC methods (AOAC, 1975); soluble and insoluble dietary fiber was assessed by the enzymatic–gravimetric procedure (Prosky L, 2000), and carbohydrates were evaluated as simple sugars (Zygmunt LC, 1982) and total starch (Champ M, 1992). L-arginine content was assessed by HPLC (Cunico R, 1986).

4.1.7 The software to decode foods

Several softwares to decode foods are present, but we could not find appropriate software that could decode the micronutrients content, in particular the aminoacids contents. Therefore we decide to customize well-known software (Nutritionist Pro 2.5, Axial System, Stafford, Texas) by introducing the contents of all amino acids for more than 800 different foods, obtained from INRAN and USDA database items. When the modified software was ready, we analyzed the content of L-arginine in our patient's diet. This software gives the possibility to analyze the food daily composition of diet, through this application we could known the energy intake, the composition of macronutrients and micronutrients.

4.1.8 Statistical analysis

All values are expressed as Mean±SD at each time interval. The patients' response to the biscuit intervention was quantified as the difference between the values obtained at the end versus the beginning of the each food preparation intervention period. The groups were defined by the sequence of biscuit administration; i.e., the group that received the L-arginine-enriched biscuits first followed by the placebo biscuits was compared with the group that was fed the biscuits in reverse order. Differences between groups were evaluated by paired data student-T-test; a p value of less than 0.05 was taken to indicate a significant difference. All analyses were performed using Statistical Package for Social Science (SPSS) version 15.0 software (SPSS Inc., Chicago, Illinois).

References

AOAC. 1975, Official methods of analysis. 12th ed. Washington DC: Association of Official Analytical Chemists.

Champ M, 1992, Determination of resistant starch in food and food products: interlaboratory study. Eur J Clin Nutr;42(Suppl 1):S51–61.

Cunico R, et al., 1986, Amino Acid Analysis using a novel automated precolum derivatizzation system. BioChromatography;1:7–14.

Hill JM et al., 2003, Circulating endothelial progenitor cells, vascular function and cardiovascular risk. N engl J Med 348:593-600

Pi J, et al., 2000, Improved method for simultaneous determination of L-arginine and its mono- and dimethylated metabolites in biological samples by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl;742:199–203.

Prosky L, et al., 1994, Determination of soluble dietary fiber in foods and food products: collaborative study. J AOAC;77:690–4.

Zygmunt LC, et al., 1982, High pressure liquid chromatographic determination of mono- and disaccharides in pre-sweetened cereals: collaborative study. J Ass Off Anal Chem;65:256–64.

4.2 RESULTS

4.2.1 Proximate composition

In the following table (Table 3) is reported the proximate composition (% wet weight) of the products administered during the study.

(%ww)	Arginine enriched Biscuit	Placebo Biscuit
Water	**17.8**	**18.4**
Ash	**1.3**	**1.4**
Protein	**6.4***	**7.4**
Starch	**34.8**	**39.8**
Sugars	**6.9**	**8.7**
Fat	**12.5**	**14.6**
Dietary fiber	**7.2**	**7.4**
Arginine	**11.3**	**nd**
Energy (Kcal) (KJ)	**305 (1276)**	**350 (1486)**

Table 3: proximate composition of tested biscuits.
*protein content has been estimated from total N content minus N from Arginine.

During a test, the subjects consumed a 60g (180 kcal) portion of the L-arginine-enriched biscuits containing 6.6g L-arginine, 25.0 g carbohydrates, 3.8 g protein, 7.5 g fat and 4.3 g dietary fiber.

4.2.2 Metabolic variables

In the group receiving L-arginine-enriched biscuits, fasting L-arginine levels were quite double compared to the group receiving biscuits non containing L-arginine **(**117.8±26.9 vs 59.3±21.6 μmol/l; $p<0.001$) (Figure 2A), while the plasmatic levels of ADMA were lower in the group receiving the biscuits containing L-arginine as compared to the group receiving the biscuits not containing L-arginine (ADMA 0.27±0.07 vs 0.32±0.06 μmol/l; $p< 0.004$) (Figure 2B). We, also, calculated the L-arginine/ADMA ratio and we found that it was higher in the group receiving biscuits containing 6.6 g of L-arginine than in the group receiving biscuits non containing L-arginine (436.3 17.7 vs 185.3 27.9; $p<0.001$). (Figure 2C).

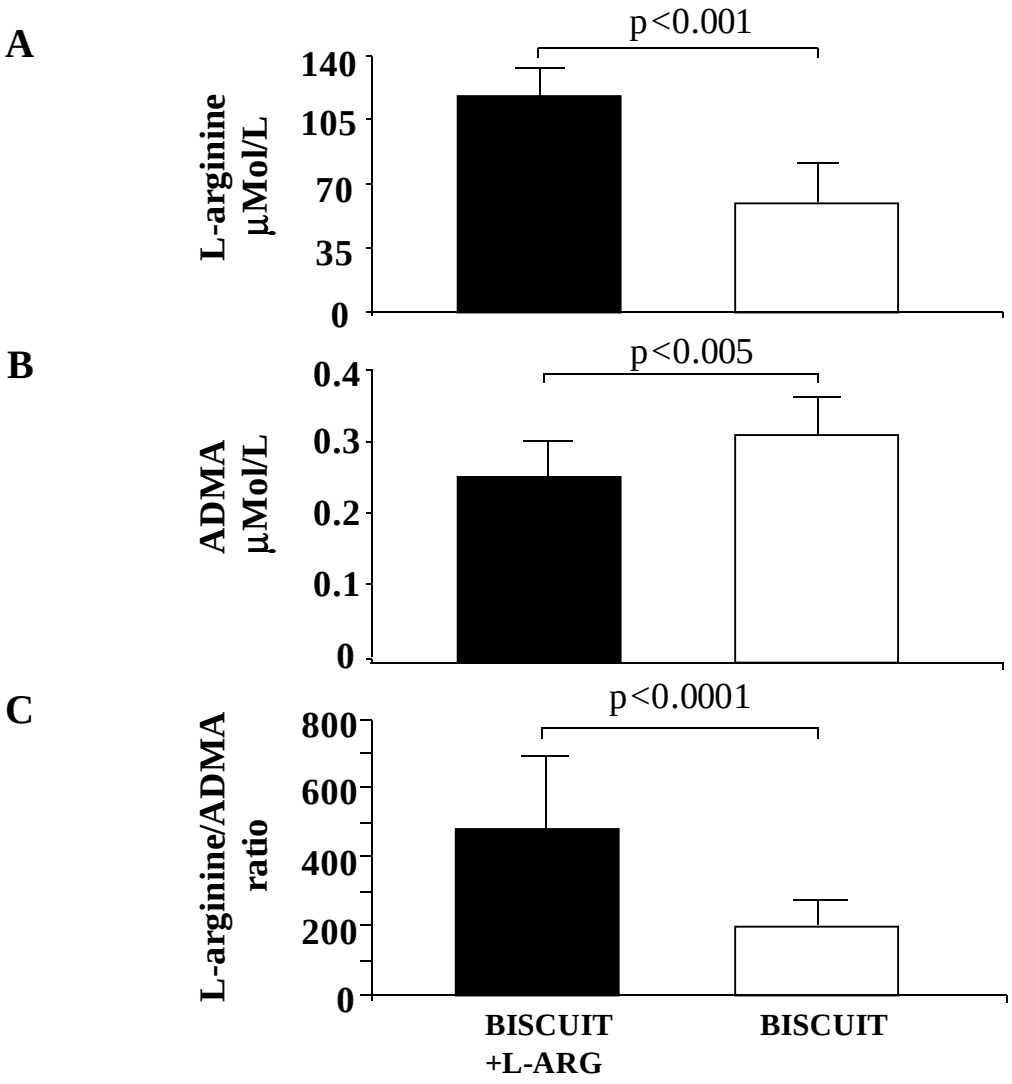

Figure 2: A. Fasting L-arginine levels in subjects receiving the L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **B**. Fasting ADMA levels in subjects receiving the L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **C**. Ratio of L-arginine/ADMA levels in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). Data are presented as Mean±SD.

The evaluated increase of L-arginine availability in treated subjects corresponded to a significant increase in nitric oxide (NOx) and cGMP levels during OGTT. In particular, nitric oxide (NOx) and cGMP plasma levels were significantly higher in the group receiving biscuits with L-arginine as compared to the group receiving placebo biscuits (Figures 3A and 4A). Area under the curve (AUC) and incremental area under the curve (ΔAUC), calculated geometrically for NOx and cGMP respectively, were also evaluated. AUC NOx (1250±200 vs 730±185 µmol/L* 120 min; $p<0.05$) and ΔAUC cGMP (2495±329 vs 1742±155 pmol/mL* 120 min; $p<0.05$) were significantly increased when compared to the group receiving placebo biscuits (Figure 3B and 4B).

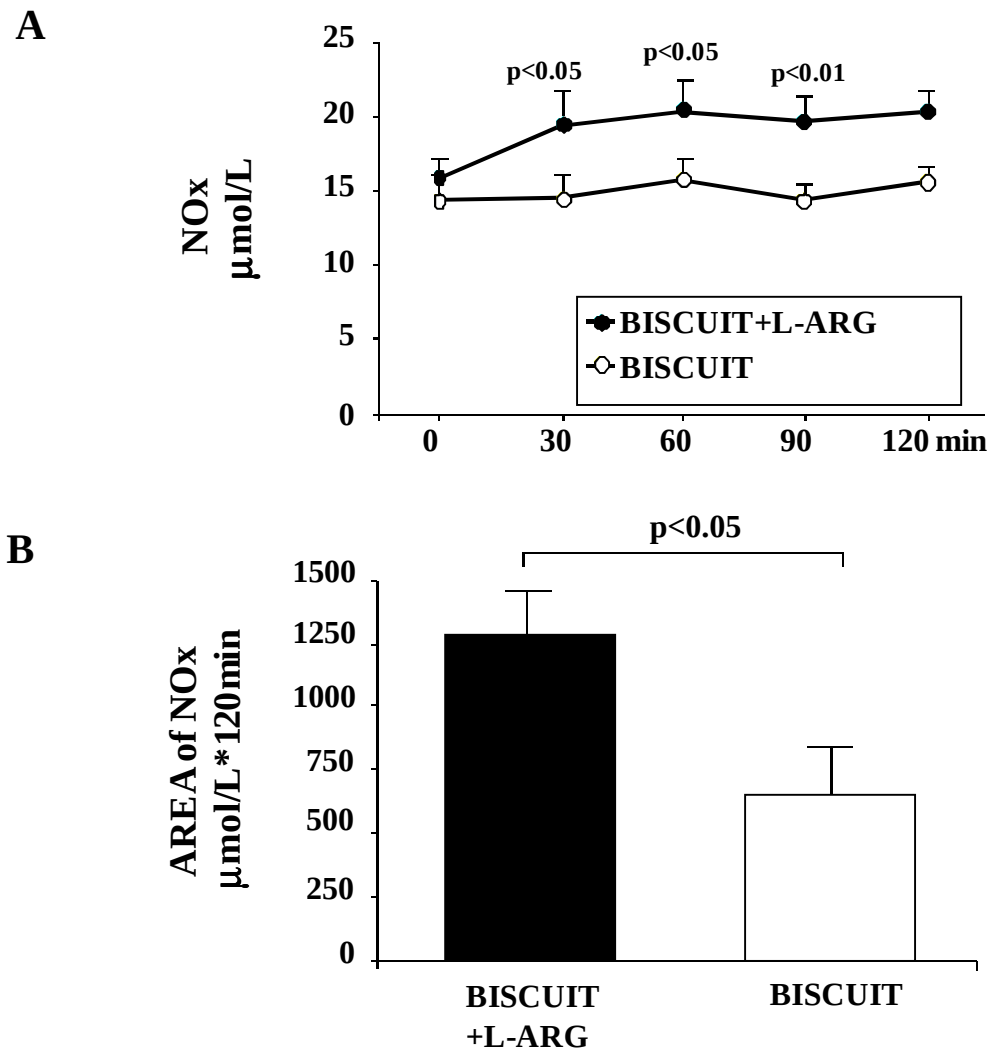

Figure 3: A. Patterns of plasma NOx levels during OGTT in subjects receiving L-arginine-enriched biscuits (black circles) and in subjects receiving placebo biscuits (white circles). **B**. Comparison of the areas under the curve for NOx (AUC NOx) during the OGTT in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram), calculated using the trapezoidal rule. Data are presented as Mean±SD.

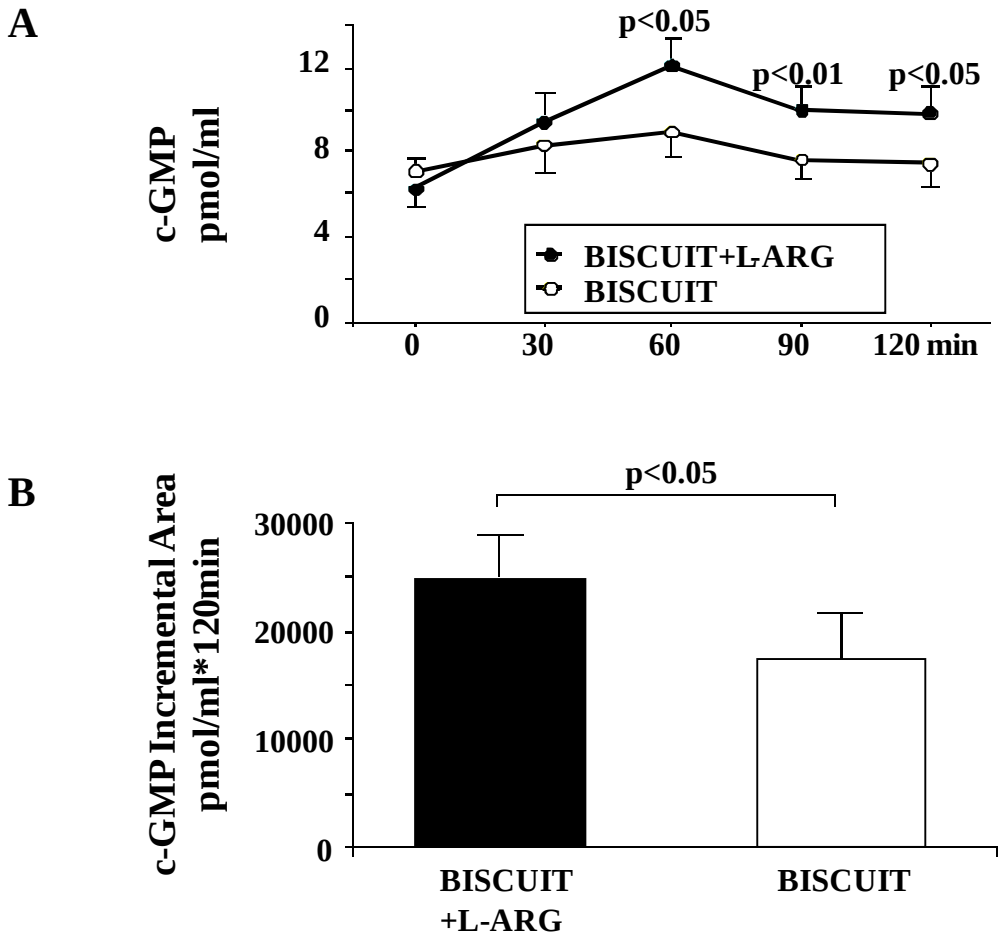

Figure 4: A. Patterns of plasma cGMP levels during OGTT in subjects receiving L-arginine-enriched biscuits (black circles) and in subjects receiving placebo biscuits (white circles). **B.** Comparison of the incremental areas under the curve for cGMP (ΔAUC cGMP) during OGTT in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram), calculated using the trapezoidal rule. Data are presented as Mean±SD.

Post-ischemic blood flow significantly ($p<0.01$) increased in the group receiving L-arginine enriched biscuits as compared to the group receiving placebo, suggesting a functional effect of the amino acid added to the food product (Figure 5).

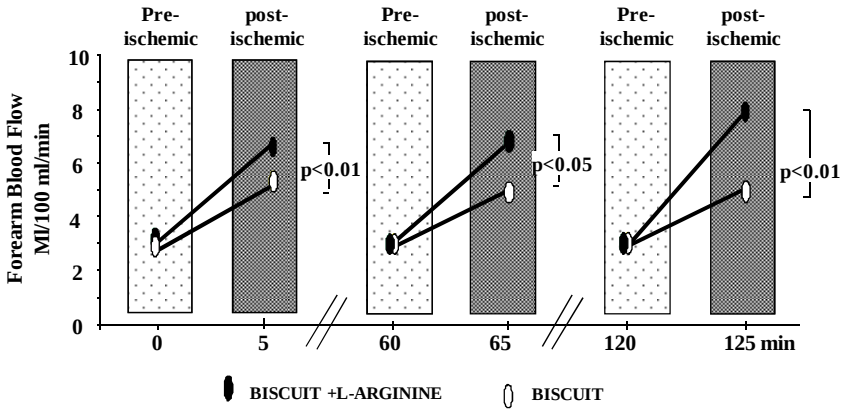

Figure 5: Incremental increase from basal levels in post-ischemic blood flow in subjects receiving the two different treatment conditions. Data are presented as Mean±SD.

The endothelial progenitor cells, evaluated as both KDR/CD34 circulating cells than as colony-forming units at the end of the cell culture, were higher in the group receiving the biscuits containing L-arginine as compared to the group receiving placebo biscuits (KDR/CD34 291.6±134.7 vs 162.9±81.4; p<0.001; CFUs 8.3±6.2 vs 2.9±2.3; p<0.01) (Figure 6A and B).

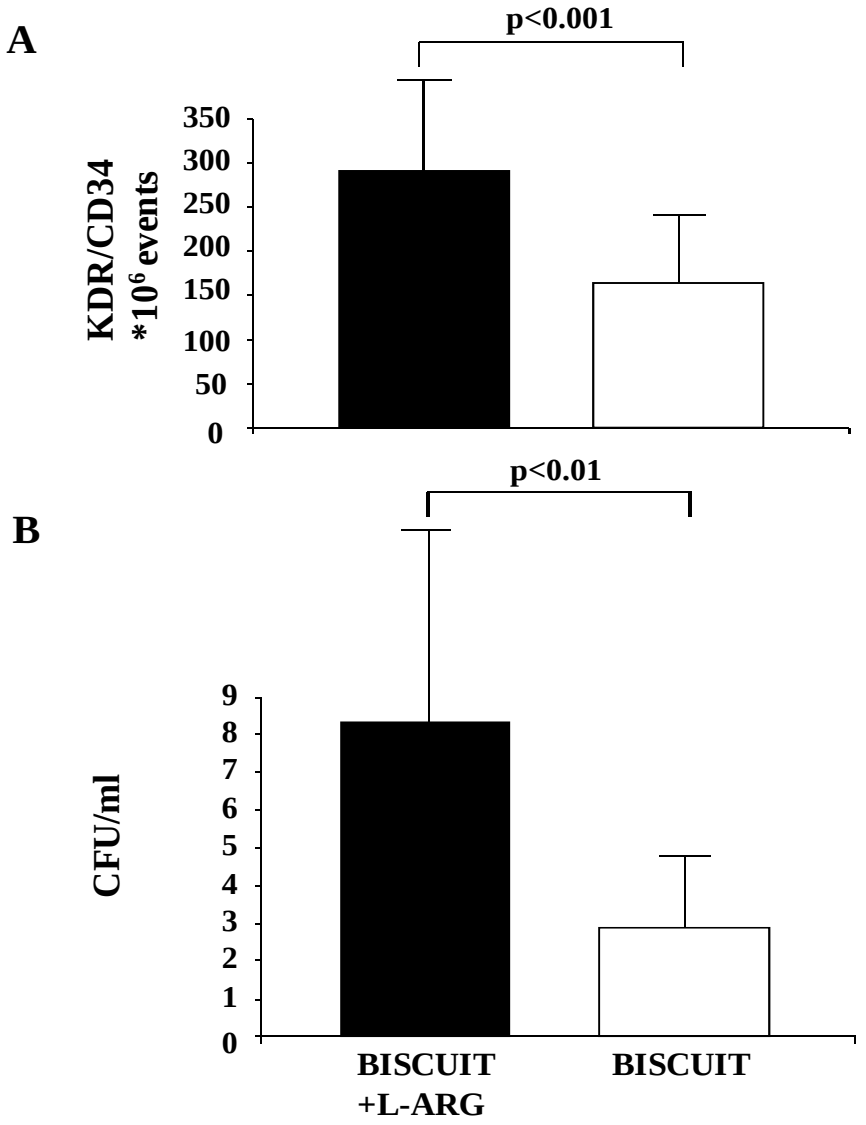

Figure 6: A. KDR/CD34 circulating cells in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects placebo biscuits (white histogram). **B**. Colony-forming-units (CFUs) in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). Data are presented as Mean±SD.

Very interesting, the molecules involved in the EPCs mobilisation and homing, evaluated both as peripheral plasmatic levels and as gene expression levels, were higher in the group receiving L-arginine-enriched biscuits than in the group receiving placebo biscuits. In particular VEGF-α was 51.5±17.2 vs 38.1±10.9 pg/ml; $p<0.05$; SCF (sKit-L) 1179.1±161 vs 1140.1±163 pg/ml: $p< 0.05$; MMP-9 29.2±12.9 vs 19.4±7.7 ng/ml; $p<0.01$; SDF-1α 2931.2±510 vs 2561.3±349 pg/ml; $p<0.01$, respectively (Figure 7A-D). While the expression of Akt and eNOS genes were 2.1±2.2 vs 1.6±2.2 ΔΔct; $p<0.01$ and 3.3±2.9 vs 1.1±0.9 ΔΔct; $p<0.05$ respectively (Figure 8A and B).

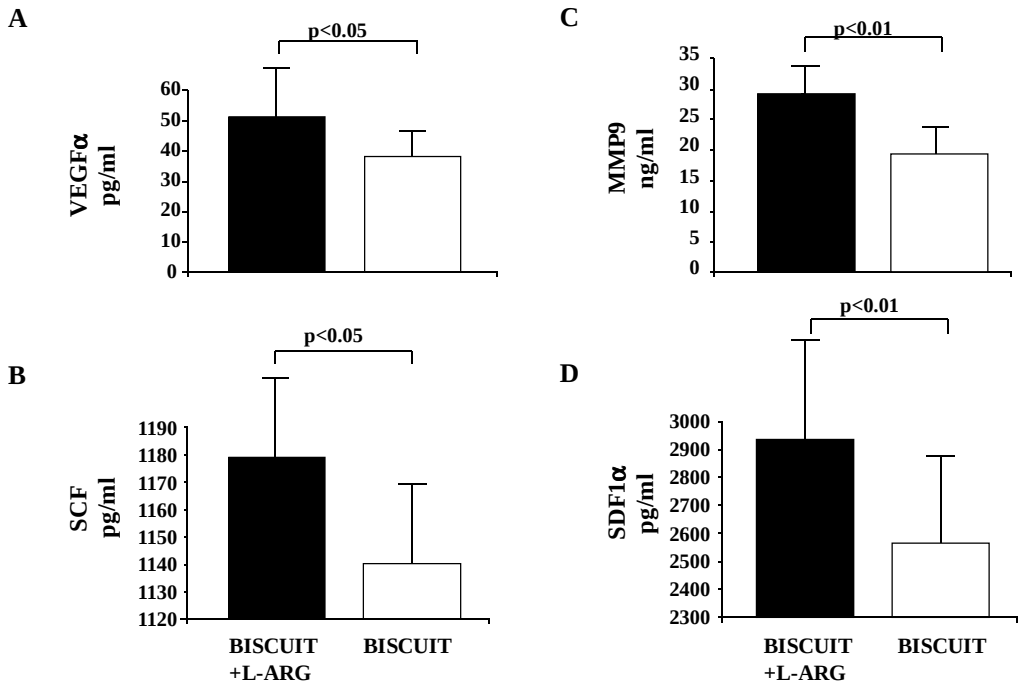

Figure 7: A. Fasting VEGF-α levels in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **B.** Fasting SCF (sKit-L) levels in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **C.** Fasting MMP-9 levels in subjects receiving the L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **D.** Fasting SDF-1α levels in subjects receiving the L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). Data are presented as Mean±SD.

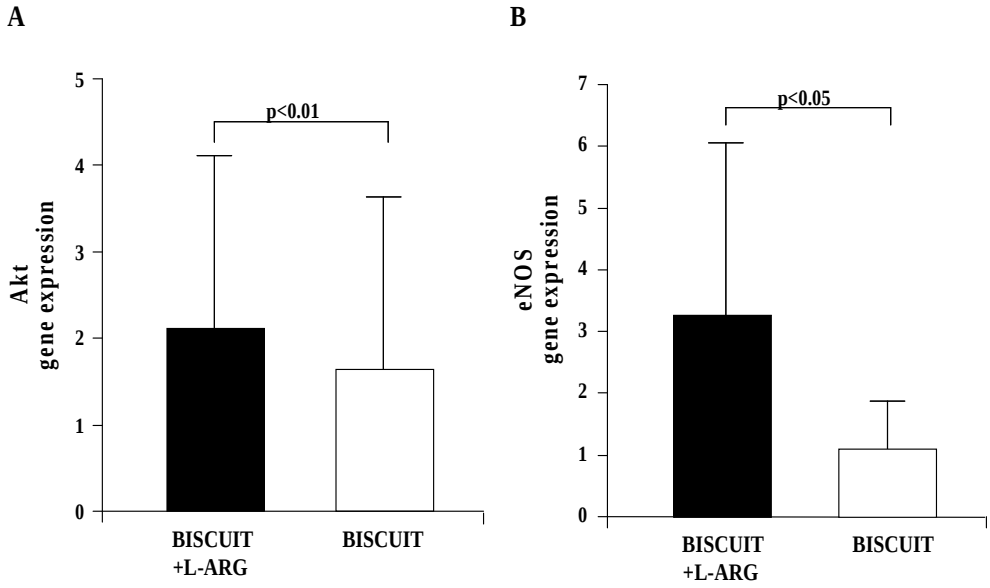

Figure 8: **A**. Akt gene expression levels (calculated with the formula $2^{-\Delta\Delta CT}$; see materials and metods 4.1.4) in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). **B**. eNOS gene expression levels in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). Data are presented as Mean±SD.

Interestingly glucose levels and AUC of glucose were significantly lower in the group receiving the biscuits containing L-arginine as compared to the group receiving the Placebo biscuits, even if insulin levels were not significantly different (Figure 9 A-D).

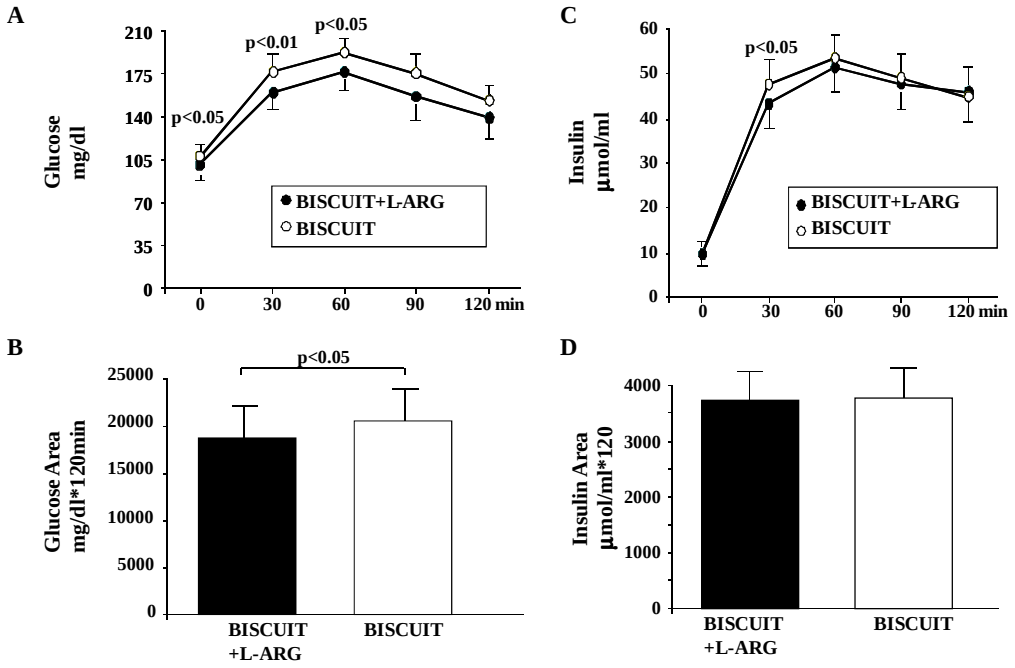

Figure 9: A. Patterns of plasma glucose levels during OGTT in subjects receiving L-arginine-enriched biscuits (black circles) and in subjects receiving placebo biscuits (white circles). **B**. Comparison of the areas under the curve for glucose (AUC glucose) during the OGTT in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram), calculated using the trapezoidal rule. **C.** Patterns of plasma insulin levels during OGTT in subjects receiving the L-arginine-enriched biscuits (black circles) and in subjects receiving placebo biscuits (white circles). **D.** Comparison of the areas under the curve for insulin (AUC insulin) during the OGTT in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram), calculated using the trapezoidal rule. Data are presented as Mean±SD.

In the group receiving L-arginine enriched biscuits body weight was reduced by 2.57±0.33 kg as compared to a body weight reduction of 1.37±0.34 assessed after 14 days biscuits without L-arginine ($p<0.05$). Interestingly, during the 14 days of food intervention with L-arginine, quite all the body weight changes related to a reduction of fat mass (2.02±0.52 kg vs 0.70±0.50 kg ; $p<0.01$). Conversely, no differences were demonstrated in the loss of fat free mass between the two groups (figure 10).

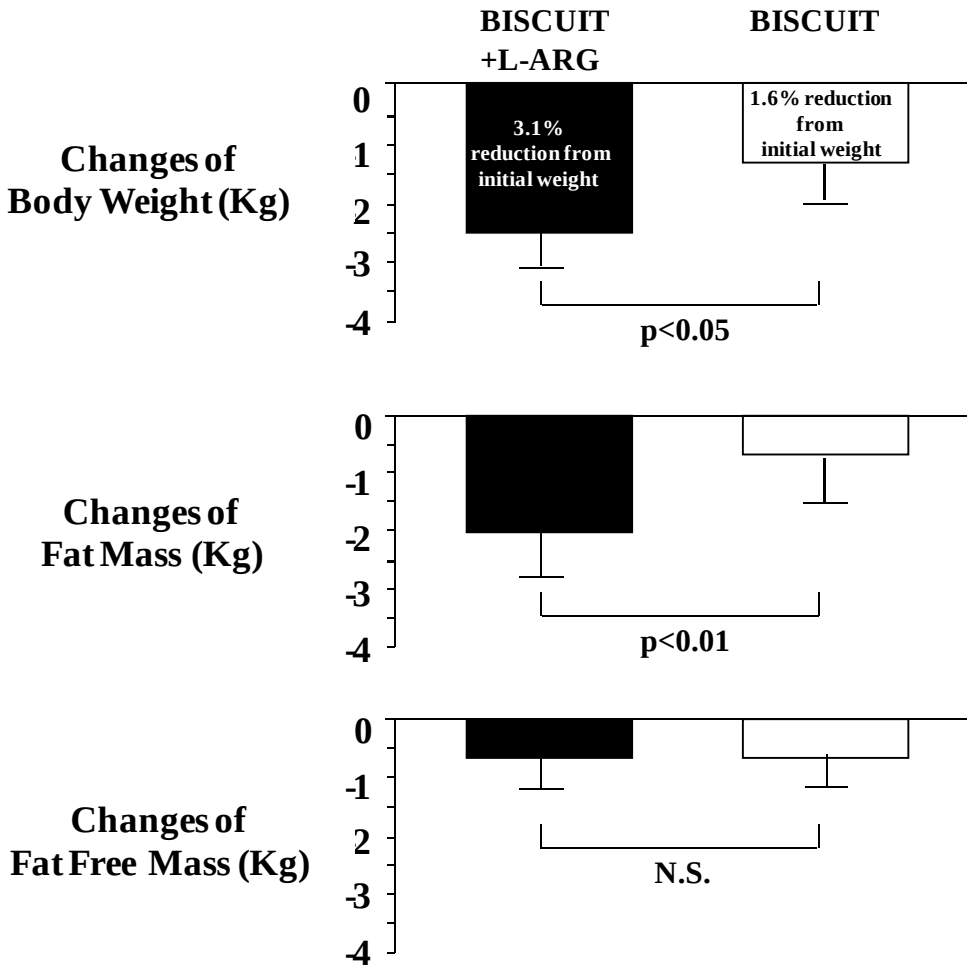

Figure 10: Changes in body weight, fat mass and fat free mass as compared to baseline in subjects receiving L-arginine-enriched biscuits (black histograms) and in subjects receiving placebo biscuits (white histogram). To evaluate changes in body weight compositions, patients' response to food interventions was calculated as the difference between the values obtained at the end and at the beginning of the each food preparation intervention period. Data are presented as Mean±SD.

4.3 DISCUSSION

In the present study, a new food product, in form of a biscuit obtained by a novel technological process (aggregation by sonication at low temperature), comprising a high content in L-arginine and a low content in sugars and proteins, was used in obese subjects with impaired glucose tolerance and metabolic syndrome.

It is noteworthy that the present L-arginine-enriched biscuit composition is in accordance with current dietary guidelines.

Regarding total carbohydrates content, the food product appears interesting not only for its low carbohydrate content, but also for the quality of carbohydrate itself. In fact, in line with current dietary guidelines supporting a limited sugars intake up to 12% of daily energy in favour of complex carbohydrates (starch), L-arginine-enriched biscuit contains low amount of sugars (about 6.9 % ww) and mainly starch from whole cereals (35 % ww). This feature is favourable in the light of the potential low glycemic impact induced by whole-grain cereal starch and it is particularly advantageous since the present food preparation was used in subjects at high risk of cardiovascular disease.

The interesting results on an amelioration of endothelial function, insulin sensitivity and reduction of adipose tissue are in line with previous results. Fu et al. studied Zucker Diabetic fatty rats submitted to a dietary supplementation of drinking water containing L-arginine (1.51%) or alanine (2.55%, isonitrogenous control) for 10 weeks. Arginine supplementation significantly reduced weight of retroperitoneal and epididymal adipose tissue by 45 and 25%, respectively, as well as circulating levels of glucose by 25%, triglycerides by 23%, free fatty acids by 27%, homocysteine by 26%, ADMA by 18-21% and leptin by 32%. Further, in this manuscript results of the microarray analysis indicated that Arginine supplementation increased adipose tissue expression of key genes responsible for fatty acid and glucose oxidation, NO synthase-1, AMP-activated protein kinase and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (Fu WJ).

In the same line the results of amelioration of endothelium-dependent vasorelaxation were previously reported. Hayashi et al. demonstrated rabbits fed a high-cholesterol diet that an 12 weeks oral administration of L-arginine plus L-citrulline, either alone or

in combination with antioxidants, caused a marked improvement in endothelium-dependent vasorelaxation and blood flow, dramatic regression in atheromatous lesions, and decrease in superoxide production and oxidation-sensitive gene expression (Hayashi T). In healthy young subjects L-arginine infusion at 10, 20, 40, and 60 mg/min increase forearm blood flow dose dependently, moreover this effect was amplified by the simultaneous infusion of acetylcholine (Imaizumi T). Also Bode-Boger et al. investigated healthy volunteers and the effect of an intravenous infusion of L-arginine (a dose of 30 g for 30 min) on blood pressure; they showed that L-arginine reduced blood pressure and that this effect was strikingly evident for diastolic pressure (Boger RH). Creager et al. demonstrated that in hypercholesterolemic patients an acute administration of L-arginine (10 mg/kg/min i.v.) was able to induce an improvement of L-arginine blood vessels endothelium-dependent vasodilation (Creager MA). Egashira et al. examined the effect of L-arginine on endothelium-dependent coronary vasodilation in patients with microvascular angina throughout an intracoronary infusion of L-arginine (50 mg/mm) on acetylcholine-induced coronary vasomotion. L-arginine was able to improve endothelium-dependent vasodilation of coronary microcirculation in these patients (Egashira K). An acute infusion of low dose of L-arginine infusion (0.125 g/min) at dose not able to stimulate the insulin-induced endothelial dependent vasodilation, increased forearm blood flow, cyclic-GMP forearm release and decreased endothelin-1 levels in healthy subjects. As shown by our group, in patients affected by microvascular angina similar amount of L-arginine infusion increased forearm blood flow, circulating NOx and forearm cGMP release and decreased endothelin-1 levels and systolic and diastolic pressure. Moreover, the pre-infusion of low-dose of L-arginine restored the endothelin-1, NOx, forearm cGMP release responses after insulin bolus in these patients (Piatti P).

In addition, when endothelial function is impaired as in healthy very old age subjects, oral L-arginine supplementation was able to improved endothelial vasodilation (Bode-Boger SM). Clarkson et al. found that L-arginine addition improved endothelium-dependent vasodilation compared to placebo in a double blind cross over study with 4 weeks L-arginine supplementation (7 g three times a day) or

placebo in hypercholesterolemic young adults (Clarkson P). Hambrecht et al. studied the effect of L-arginine (8 g daily) and physical exercise in patients with chronic heart failure. After 4 weeks both L-arginine than physical exercise were able to improve endothelium dependent vasodilation, but when associated L-arginine plus exercise produced an additive beneficial effects on endothelium dependent vasodilation (Hambrecht R). Finally, Palloshi et al. demonstrated that a chronic administration of L-arginine (4 weeks, 6 g daily) in patients with hypertension and microvascular angina determined an improvement of endothelial function and a significant amelioration of symptoms (Palloshi A).

In 1992, Vallance et al. first described the presence of asymmetric dimethylarginine (ADMA) as an endogenous inhibitor of eNOS in human plasma and urine (Vallance P). ADMA inhibits vascular nitric oxide production within the concentration range found in patients with vascular disease. Elevated ADMA concentrations are present in patients with hypercolesterolemia, hyperhomocisteinemia, diabetes mellitus, insulin resistance, atherosclerosis and hypertension, cardiovascular disease and chronic heart failure (Abbasi F). In the present study, the administration of L-arginine was able to decrease ADMA levels, increasing L-arginine/ADMA levels.

A quite important and new result of the present study was that we were able to demonstrate, for the first time, that L-arginine enriched biscuits increased the number of endothelial progenitor cells evaluated both as circulating peripheral cells and as colony-forming-units after cell culture. One possible explanation could be related to a decrease in glucose levels and to an improvement of insulin sensitivity and secretion in subjects receiving L-arginine enriched biscuits compared to placebo. In fact, it has been demonstrated that disorders in glucose regulation are associated with abnormalities in EPC biology, including their reduced circulating number, defective mobilization from bone marrow and impaired functional properties such as their capacity to mediate endothelial repair. Fadini et al., in 2006, showed a reduced number of EPCs in individuals with impaired glucose tolerance compared with those normal glucose regulation and in a different study they displayed that EPCs negatively correlated with components of metabolic syndrome and with HOMA-IR, a score of

insulin resistance. (Fadini GP 2006). EPCs were also found to be lower in another study of obese men with the metabolic syndrome compared with non-obese healthy controls (Westerwell PE). Thus, reduced circulating EPCs may be attributable to a number of factors, including defective mobilization, decreased proliferation and shortened survival in the circulation (Aicher A, 2003; Aicher A, 2004; Hristov M).

Another explanation which could concur with alteration in glucose metabolism/insulin resistance is that reduced NO bioavailability impairs EPC mobilization and function in experimental models. (Balletshofer BM; Duncan ER; Wheatcroft SB; Steinberg HO). Insulin resistance is closely associated with abnormalities in NO bioavailability and PI3K/Akt signalling, both of which play a crucial role in EPC mobilization from the bone marrow (Aicher A; Hristov M; Dimmeler S; Thum T; Werner C; Urao N) through the activation of eNOS. This is related to the fact that insulin resistance impairs the insulin binding to its receptor, impairing the cascade of protein phosphorilation, involving the signalling intermediates (IRSs, PI3K and Akt). The alteration of the pathway results in both, altered traslocation of GLUT4 to the cell membrane, which mediates glucose uptake, and reduced phosphorilation of eNOS culminating in increased NO production.

The results of increased expressions of eNOS and Akt genes in EPCs from subjects receiving L-arginine enriched biscuits are, in our opinion, of particular interest, suggesting that L-arginine is able to enhance the expression levels of genes involved, non only in metabolic and endothelial function, but also in EPCs mobilization and function. Physical exercise, statins also mobilize EPCs from the bone marrow via a partially NO-dependent mechanism, which is abolished by concomitant treatment with the NO synthase inhibitor l-NAME (*N*G-nitro-l-arginine methyl ester) (Laufs U, 1998; Laufs 2004). Fadini et al. reported that DDp-4 inhibitor, Sitagliptin, was able to increase EPCs cells by the role of SDF-1α. (Fadini GP, 2010). Also in the present study, SDF-1α levels increased suggesting that L-arginine could follow, at least in part, the same pathway of DPP-4 inhibitor in increasing EPCs levels.

Finally, we were able to demonstrate that L-arginine enriched biscuits is able to increase VEGF-α, MMP-9 and SCF (sKit-L), which are peripheral markers of EPCs

mobilization. In fact, mobilization of EPCs from the bone marrow into the circulation occurs in response to growth factors and cytokines, including VEGF (vascular endothelial growth factor), SDF-1α (stromal-cell-derived factor-1α) (Aicher A, 2005). Both VEGF and SDF-1α up-regulate bone marrow MMP-9 (matrix metalloproteinase-9) activity, which cleaves the progenitor cell membrane-bound kit ligand, allowing mobilization of progenitors into the bone marrow vascular zone (Aicher A, 2005). Nitrosylation of MMP-9 by NO, released from bone marrow stromal cells, is required for VEGF-stimulated EPC mobilization (Aicher A, 2005). Chemokine signalling plays a major role in directing circulating progenitor cells to sites of injury.

An interesting result of this study, conducted in obese subjects with IGT and MS, was the significant decrease of body weight in the group receiving the biscuits added with L-arginine which was quite completely accounted by a loss of fat mass. These results corroborates previous data in which oral administration of L-arginine added to a structured physical activity and hypo caloric regimen for 21 days was able to decrease body weight mainly as a reduction of fat mass sparing fat free mass in obese type 2 diabetic subjects (Lucotti P, 2006). The strength of the present study is that the loose of body weight was achieved without the help of a structured program of physical activity in patients quite sedentary. Moreover, the obese subjects admitted to take the biscuits with L-arginine loosed more weight and fat mass that the same subjects taking the biscuits without the addition of L-arginine. Recently Monti LD et al. performed a mono-centre, randomized, double-blind, parallel-group, placebo-controlled, phase III trial (named l-arg trial). In this study, 144 individuals, affected by impaired glucose tolerance (IGT) and metabolic syndrome (MS), received 6.4g/day of l-Arg, or placebo for 18 months plus a 12-month extended follow-up period after study drug termination, in order to prevent or delay type 2 diabetes and to normalize glucose tolerance in individuals at high risk for type 2 diabetes. The results showed that the supplementation of l-arg for 18 months does not significantly reduce the incidence of diabetes but does significantly increase regression to NGT. (Monti LD, 2012).

The results of the present study are particularly interesting mainly on the action of L-arginine on EPCs, Akt/eNOS activation, EPCs increment and cytochine/chemokine

modulation. Interestingly, the results are similar to those obtained with a pharmacological approach using a DPP-4 inhibitor, Sitagliptin in patients with type 2 diabetes mellitus. Since in the long-term study, L-arginine had no adverse events, as demonstrated by Monti et al.(2012), it is possible to suggest the use of this food product in subjects with glucose intolerance and cardiovascular disease, in addition to a usual standard care. The results of this study need to be reproduced in an increased number of subjects for a prolonged period of time. However, if these results are replicated a new nutritional approach could be easily achieved, which is safe, cost-saving and could be utilized by a large number of subjects.

References

Abbasi F, et al., 2001, Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. Am J Cardiol 88:1201-1203.

Aicher A, et al., 2003, Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat. Med. 9, 1370–1376

Aicher A, et al., 2004, The role of NOS3 in stem cell mobilization. Trends Mol. Med. 10, 421–425

Aicher A et al., 2005, Mobilizing endothelial progenitor cells. Hypertension 45, 321–325

Balletshofer BM, et al., 2000, Endothelial dysfunction is detectable in young normotensive first-degree relatives of subjects with type 2 diabetes in association with insulin resistance. Circulation 101, 1780–1784

Bode-Boger SM, et al., 2003, Oral L-arginine improves endothelial function in healthy individuals older than 70 years. Vasc Med 8: 77-81.

Boger RH, Bode-Boger SM, 2001, The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol 41:79-99.

Clarkson P, et al., 1996, Oral L-arginine improves endothelium-dependent vasodilation of hypercholesterolemic young adults. J Clin Invest 97: 1989-1994.

Creager MA, Gallagher SJ, et al., 1992, L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest 90:1248-1253.

Dimmeler S, et al., 2001, HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J. Clin. Invest. 108, 391–397

Duncan ER, et al., 2007, Accelerated endothelial dysfunction in mild prediabetic insulin resistance: the early role of reactive oxygen species. Am. J. Physiol. Endocrinol. Metabol. 293, E1311–E1319

Egashira K, et al., 1996, Effects of L-arginine supplementation on endothelium-dependent coronary vasodilation in patients with angina pectoris and normal coronary arteriograms. Circulation 94:130-134.

Fadini GP, et al., 2006, Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. Eur. Heart J. 27, 2247–2255

Fadini GP, et al., 2007, Glucose tolerance is negatively associated with circulating progenitor cell levels. Diabetologia 50, 2156–2163

Fadini GP, et al., 2010, The Oral Dipeptidyl Peptidase-4 Inhibitor Sitagliptin Increases Circulating Endothelial Progenitor Cells in Patients With Type 2 Diabetes. Possible role of stromal-derived factor-1. Diabetes Care 33:1607–1609.

Fu WJ, 2005, Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr 135:714-721

Hambrecht R, et al., 2000, Correction of endothelial dysfunction in chronic heart failure: additional effects of exercise training and oral L-arginine supplementation. J Am Coll Cardiol 35:706-713

Hayashi T, Juliet PA, et al., 2005, L-Citrulline and L-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits. Proc Natl Acad Sci U S A. 20:3681-13686.

Hristov M, et al., 2003, Endothelial progenitor cells: mobilization, differentiation, and homing. Arterioscler. Thromb. Vasc. Biol. 23, 1185–1189

Imaizumi T, Hirooka Y, et al., 1992, Effects of L-arginine on forearm vessels and responses to acetylcholine. Hypertension 20:511-517.

Laufs U, et al., 1998, Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 97, 1129–1135

Laufs U, et al., 2004, Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. Circulation 109, 220–226

Lucotti P, et al., 2006, Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab;291:E906–12.

Monti LD, et al., 2012, Effect of a long-term oral L-arginine supplementation on glucose metabolism: a randomised, double-blind, placebo-controlled trial. Diabetes Obes Metab. May 4.

Palloshi A, et al., 2004, Effect of oral L-arginine on blood pressure and symptoms and endothelial function in patients with systemic hypertension, positive exercise tests, and normal coronary arteries. Am J Cardiol 93:933-935

Piatti PM, et al., 2001, Long-term oral L-arginine administration improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients. Diabetes Care 24:875-880

Steinberg HO, et al., 1996, Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. J. Clin. Invest. 97, 2601–2610

Thum T, et al., 2007, Age-dependent impairment of endothelial progenitor cells is corrected by growth-hormone-mediated increase of insulin-like growth-factor-1. Circ. Res. 100, 434–443

Urao N, et al., 2006, Erythropoietin-mobilized endothelial progenitors enhance reendothelialization via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia. Circ. Res. 98, 1405–1413

Vallance P, et al., 1992, Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 339: 572–575

Werner C, et al., 2007, The peroxisome proliferator-activated receptor-γ agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance. Diabetes 56, 2609–2615

Westerweel PE, 2008, Endothelial progenitor cell levels in obese men with the metabolic syndrome and the effect of simvastatin monotherapy vs. simvastatin/ezetimibe combination therapy. Eur. Heart J. 29, 2808–2817

Wheatcroft SB, et al., 2004, Preserved glucoregulation but attenuation of the vascular actions of insulin in mice heterozygous for knockout of the insulin receptor. Diabetes 53, 2645–2652

5. CONCLUSION

In conclusion the study results reveal that consumption of L-arginine-enriched biscuits with low sugar content for 14 days was safe and useful for improving endothelial and vascular function, insulin sensitivity and body mass composition.

As a new hypothesis of our study, we evaluated the role of endothelial progenitor cells in state of insulin resistance and after a nutritional intervention. In particular, we evaluated Akt/eNOS pathway in EPCs and we demonstrated that L-arginine added biscuit was able to restore EPC mobilization and differentiation properties. Moreover, L-arginine-enriched biscuits improved glucose metabolism, blood flow and decreased body weight and fat mass in subjects with IGT and MS.

APPENDIX 1

Oral communication at congress SID Torino 23-26 May 2012

VARIANTI DI ENOS E PREVALENZA DI DIABETE TIPO 2 IN UNA COORTE DI SOGGETTI NORMALI AD ALTO RISCHIO PER DIABETE

E. Galluccio[1] B. V. Fontana[1] S. Costa[1] P. Lucotti[1] E. Setola[1] A. Rossodivita[2] A. Cappelletti[2] A. Dei Cas[3] L. Franzini[3] A. Margonato[2] O. Alfieri[2] E. Bosi[1] I. Zavaroni[3] P. Piatti[1] L. Monti[1]

[1]Divisione di Scienze Metaboliche e Cardiovascolari e Dipartimento di Medicina Interna e Specialistica, Istituto Scientifico San Raffaele, Milano
[2]Dipartimento Cardio-Toraco-Vascolare, Istituto Scientifico San Raffaele, Milano
[3]Dipartimento di Medicina Interna e Scienze Biomediche, Università di Parma, Parma

Precedentemente abbiamo riportato un'associazione genetica fra 2 SNPs di eNOS con diabete tipo 2 (DMT2) e sindrome metabolica in pazienti diabetici di tipo 2. Nel presente studio, abbiamo valutato l'effetto di 4 SNPs di eNOS sulla regolazione del metabolismo glucidico e sulla secrezione insulinica in soggetti ad alto rischio di sviluppare la patologia, first degree relatives (FDR) di DMT2. La popolazione consisteva di 1274 soggetti (M 813 e F 461) con età media 57.5±11.5 anni. Circa il 50% dei soggetti presentava uno o più fattori diagnostici per sindrome metabolica. Variabili cliniche e metaboliche sono state misurate al basale e durante OGTT. La discriminazione allelica è stata valutata su 4 SNPs di eNOS: rs1799983 sull'esone 7, rs753482 sull'introne 18, rs743506 sull'introne 19 e rs373001020 sull'introne 20. La presenza dei quattro alleli mutati associava con aumentati livelli di glicemia e di insulinemia durante OGTT, con un incremento di HOMA-IR e una riduzione del Matsuda e Disposition Index. La prevalenza di DMT2 era significativamente aumentata nei portatori di alleli mutati rispetto ai portatori di alleli non mutati; con un OR per DMT2 di 2.55 (CI 95%:1.78-3.66) per l'allele mutato di rs1799983 e di 3.42 (CI 95%: 2.32-5.05) per l'allele mutato di rs753482. Sono stati definiti quattro aplotipi maggiori (GAAT, TCGC, TAAT e GCGC). Haplo TCGC aveva una frequenza maggiore in soggetti con impaired glucose regulation (IGT+DMT2 all'OGTT), mostrando livelli glicemici e insulinemici durante OGTT significativamente più elevati, un aumentato HOMA-IR e ridotti Matsuda e Disposition Index rispetto agli altri aplotipi. In conclusione, la presenza di 4 SNPs di eNOS sembra essere associata con una maggiore prevalenza di impaired glucose regulation in una coorte di soggetti non diabetici noti, FDR di DMT2

Oral communication at the congress GdS Padova febbraio 2012

ASSOCIAZIONE FRA POLIMORFISMI DI eNOS E PREVALENZA DI DIABETE TIPO 2 IN UNA POPOLAZIONE DI SOGGETTI NORMALI AD ALTO RISCHIO PER SVILUPPARE LA PATOLOGIA .

E Galluccio[1], BV Fontana[1], S Costa[1], P Lucotti[2], E Setola[2], A Rossodivita[3], A Cappelletti[3], A Dei Cas[4], L Franzini[4], A Margonato[3], O Alfieri[3], E Bosi[2], I Zavaroni[4], PM Piatti[2], LD Monti[1-2]
[1]Cardio-Diabetes & Core Lab. Unit, [2]Cardio-Metabolism and Clinical Trials Unit, [3]Dept. of Internal Medicine and Metabolic and Cardiovascular Science Division, San Raffaele Scientific [4]Institute, Milan and Dept. of Internal Medicine and Biomedical Sciences, University of Parma, Parma.

Il background genetico che causa diabete mellito tipo 2 e insulino resistenza non è stato ancora completamente definito. Il nostro gruppo ha riportato un'associazione genetica fra 2 polimorfismi di eNOS (Glu298Asp rs1799983G>T e rs753482-A>C) con diabete mellito tipo 2 e sindrome metabolica in una piccola coorte di pazienti diabetici. Nel presente studio, abbiamo valutato l'effetto di 4 SNPs di eNOS sulla suscettibilità di presentare un'alterata regolazione del metabolismo glucidico (alterata tolleranza ai carboidrati o diabete mellito di tipo 2 dopo carico orale di glucosio (OGTT) e sulla secrezione insulinica in soggetti ad alto rischio di sviluppare la patologia, i.e. parenti di I grado di soggetti diabetici di tipo 2, non diabetici noti. La popolazione consisteva di 1274 soggetti (M 813 e F 4619 con età media 57.5±11.5 anni, BMI 27.6±4.8 kg/m2, pressione sistolica 127±15 mmHg e pressione diastolica 79±9 mmHg. Circa il 50% dei soggetti presentava uno o più fattori diagnostici per sindrome metabolica. Variabili metaboliche, di sensibilità e di secrezione insulinica e di funzione endoteliale sono state misurate al basale e durante OGTT. La discriminazione allelica è stata valutata su 4 SNPs di eNOS (rs1799983 in posizione 150327044 ($Glu^{298}Asp$) sull'esone 7, rs753482 (A/C) in posizione 150337316 sull'introne 18, rs743506 (A/G) in posizione 150337848 sull'introne 19 e rs373001020 (T/C) in posizione 150338348 sull'introne 20. La presenza dei quattro alleli mutati associava significativamente con aumentati livelli di glicemia e di insulina durante OGTT, con un incremento dei livelli di HOMA-IR e un riduzione della sensibilità insulinica durante OGTT (Matsuda Index), e un'alterata secrezione insulinica valutata come first phase insulin secretion/HOMA-IR ratio. La prevalenza del diabete mellito tipo 2 era significativamente aumentata con valori fra il 18.5 e il 20.5% nei portatori di alleli mutati rispetto al 5.5-7.3% per i portatori di alleli non mutati; con un OR di presentare diabete mellito tipo 2 dopo OGTT di 2.553 (CI 95%:1.782-3.656) per l'allele mutato di rs1799983 e di 3.419 (CI 95%: 2.316-5.047) per l'allele mutato di rs753482. Sono stati definiti quattro aplotipi maggiori (GAAT, TCGC, TAAT e GCGC) mentre altri aplotipi rari (frequenza inferiore al 3%) sono stati raggruppati determinando ~8% del totale mentre i due aplotipi haplo.GAAT e haplo.TCGC, determinavano ~80% del totale. Haplo.TCGC ha dimostrato un aumento di 2 volte nella prevalenza di diabete di tipo 2 rispetto a haplo.GAAT con un OR di 2.100 (CI 95%: 1.725-2.557, $p< 2.8 \times 10^{-12}$). I livelli glicemici e insulinemici durante OGTT erano significativamente più elevate nei soggetti con haplo.TCGC rispetto a haplo.GAAT and haplo. TAAC ($p<0.001$), in

presenza di aumentato HOMA-IR e ridotti livelli di Matsuda index e first phase insulin secretion/HOMA-IR ratio rispetto a haplo.GAAT ($p<0.001$). In conclusione, la presenza di 4 SNPs di eNOS sembra essere associata con una maggiore prevalenza di diabete mellito tipo 2 in una coorte di soggetti non diabetici noti ma ad alto rischio di sviluppare la patologia, i.e. parenti di I grado di soggetti diabetici di tipo 2.

Poster presentation at the congress: Scientific Retreat 29-30 November 2013

A NOVEL TRUNCATED FORM OF eNOS ASSOCIATES WITH ALTERED VASCULAR FUNCTION

Elena Galluccio 1,2, Laura Cassina 1,3,4, Isabella Russo 5, Fabrizio Gelmini 6, Emanuela Setola 7, Luca Rampoldi 8, Lorena Citterio 9, Alessandra Rossodivita 10, Mikel Kamami 10, Antonio Colombo 10, Ottavio Alfieri 4,10, Marina Carini 6, Emanuele Bosi 4,7, Mariella Trovati 5, PierMarco Piatti 7, Lucilla D Monti 2, Giorgio Casari 3,4

1 EG and LC are presenting authors 2 San Raffaele Scientific Institute, Division of metabolic and cardiovascular sciences, Cardiodiabetes & core Lab; 3 San Raffaele Scientific Institute, Center for Translational Genomics and BioInformatics, Neurogenomics Unit; 4 Università Vita-Salute San Raffaele; 5 Department of Clinical and Biological Sciences, Internal Medicine Unit, University of Turin, San Luigi Gonzaga Hospital, Orbassano, Italy; 6 Department of Pharmaceutical Sciences "Pietro Pratesi," Università degli Studi di Milano, Milan, Italy; 7 San Raffaele Scientific Institute, Division of metabolic and cardiovascular sciences, Cardio-metabolism and clinical trials; 8 San Raffaele Scientific Institute, Division of Genetics and Cell biology, Molecular genetics of renal disorders Unit; 9 San Raffaele Scientific Institute, Division of Genetics and Cell biology, Genomics of renal diseases and hypertension Unit; 10 San Raffaele Scientific Institute, Cardio-thoracic-vascular Department.

Nitric oxide (NO) plays a key role in vascular homeostasis and is produced by endothelial NO synthase (eNOS), encoded by NOS3 gene. We previously reported the genetic association between NOS3 rs753482-A>C polymorphism on intron 19 and coronary artery disease (CAD). In the attempt of conferring functional implication to the rs753482-A>C polymorphism, we investigated its influence on transcript maturation.

We identified three alternatively spliced NOS3 isoforms lacking exon 20 or 21 or both. All these alternative splicing events caused a frame shift and a premature stop codon insertion. Interestingly, one of the resulting truncated isoform, the transcript variant skipping exons 20-21, is translated in a novel and stable truncated form of eNOS (D20-21 eNOS). This truncated eNOS displays increased basal NO production, is insensitive to calcium stimulation, and, upon heterodimerization with the full-length eNOS protein, exerts a dominant-negative effect on NO production. The D20-21 eNOS isoform is prevalent in carriers of the rs753482-C allele. Interestingly, CAD patients and healthy subjects carriers of the rs753482-C genotype are characterized by increased NO basal levels in peripheral blood and platelets, and negatively respond to oral glucose load by failing to increase NO synthesis following insulin wave. Furthermore, forearm vasodilation after reactive hyperemia is dramatically impaired in

rs753482-C carriers. Therefore, we propose that carriers of the rs753482-C genotype are subjects at risk for the broad group of disorders originating from dysfunctional endothelium. These data open to new intriguing perspectives for different diseases involving vasculature response to NO, though the molecular mechanisms of action and regulation of the D20-21 eNOS both in CAD patients and in 82C healthy subjects need further investigation.

Poster presentation at the congress: Scientific Retreat 29-30 November 2013

TRANSLATIONAL STUDIES IN eNOS POLYMORPHISMS, INFLAMMATION AND HEALTHY NUTRITION IN THE PREVENTION OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASE

Monti Lucilla, **Galluccio Elena**, Emanuela Setola, Costa Sabrina, Fontana Barbara, Stuccillo Michela, Crippa Valentina Giulia, Bosi Emanuele, PierMarco Piatti.
Cardio-Diabetes and Core Lab., Cardio-Metabolism and Clinical Trials Unit, Cardio-Diabetes Program, Internal Medicine Department and Metabolic and Cardiovascular Science Division, San Raffaele Scientific Institute**.**

Our group is mainly involved in the -Genetic of type 2 diabetes mellitus (T2DM), -Inflammation and -Nutrition. We have a strict collaboration with the CardioThoracoVascular, Neurology, Translational Genomics Depts. We demonstrated that *NOS3* 82A/C polymorphism generates a novel and stable truncated eNOS with increased basal NO synthesis activity, failing to respond to calcium-ionomycin and insulin stimulation. We propose that the polymorphism, acting through the truncated isoform, gives endothelial dysfunction (ED) not only in cardiovascular (CV) patients but also in healthy subjects (HS). In the light of previous results, we evaluated a cohort of more than 1000 first degree relatives of T2DM, demonstrating that subjects carrying the polymorphism had a high degree of IR and β-cell dysfunction and increased risk to develop T2DM. Since, it remains unclear whether in HS with normal glucose tolerance (NGT) different degrees of insulin resistance (IR) associates with a variation in the number of circulating EPCs, we demonstrated that reduced EPCs correlated with the cluster of abnormalities linked to the IR, suggesting a possible role for EPCs as early markers of glucose intolerance and CV risk. In another study, we observed that patients with limb ischemia carrying the polymorphism had an increased risk of major cardiovascular events that correlated with lower circulating EPCs. A translational aspect of our research is the evaluation of the effects of L-arginine, a precursor of nitric oxide by eNOS. In subjects with IGT and MS, the use of 6.4 g L-arg daily when added to lifestyle intervention for 18 months significantly ameliorated glucose metabolism, reverting IGT to NGT. The consumption of L-arginine added in biscuits with a low content in sugars and proteins for 14 days improved ED, IR and insulin secretion in HS with IGT and MS. Therefore, L-arginine enriched food may have a role in the dietary management of individuals at risk of T2DM and CV. All in all, our data demonstrate that an eNOS polymorphism seems to have a functional effect giving increased ED and IR while L-arginine supplementation demonstrates beneficial effects on IR,

inflammation and ED. In both cases EPCs seem to have a predominant effects. Molecular mechanisms are actually under evaluation.

Pubblications

Galluccio E, Cassina L, Russo I, Gelmini F, Setola E, Rampoldi L, Citterio L, Rossodivita A, Kamami M, Colombo A, Alfieri O, Carini M, Bosi E, Trovati M, Piatti P, Monti LD, Casari G.
A novel truncated form of eNOS associates with altered vascular function.
Cardiovasc Res. 2013 Dec 29, on line pubblication

Monti LD, Casiraghi MC, Setola E, **Galluccio E**, Pagani MA, Quaglia L, Bosi E, Piatti P. L-arginine enriched biscuits improve endothelial function and glucose metabolism: a pilot study in healthy subjects and a cross-over study in subjects with impaired glucose tolerance and metabolic syndrome. Metabolism. 2013 Feb;62(2):255-64.

Piatti PM, Marone E, Mantero M, Setola E, **Galluccio E**, Lucotti P, Shehaj E, Villa V, Perticone F, Venturini M, Palini A, Airoldi F, Faglia E, Del Maschio A, Colombo A, Chiesa R, Bosi E, Monti LD. Effect of normalization of fasting glucose by intensified insulin therapy and influence of eNOS polymorphisms on the incidence of restenosis after peripheral angioplasty in patients with type 2 diabetes: a randomized, open-label clinical trial. Acta Diabetol. 2013 Jun;50(3):373-82.
.
Monti LD, Setola E, Lucotti PC, Marrocco-Trischitta MM, Comola M, **Galluccio E**, Poggi A, Mammì S, Catapano AL, Comi G, Chiesa R, Bosi E, Piatti PM. Effect of a long-term oral l-arginine supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2012 Oct;14(10):893-900.

Lucotti P, Monti LD, Setola E, **Galluccio E**, Gatti R, Bosi E, Piatti P. Aerobic and resistance training effects compared to aerobic training alone in obese type 2 diabetic patients on diet treatment. Diabetes Res Clin Pract. 2011 Dec;94(3):395-403.