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"Research and development of nutraceuticals useful for metabolic syndrome and inflammatory diseases."

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Abstract

Metabolic syndrome (MetS) represents a cluster of metabolic abnormalities that include hypertension, central obesity, insulin resistance, and atherogenic dyslipidaemia, and is strongly associated with an increased risk for developing diabetes and atherosclerotic and nonatherosclerotic cardiovascular disease (CVD). The pathogenesis of MetS involves both genetic and acquired factors that contribute to the final pathway of inflammation that leads to CVD. MetS has gained significant importance recently due to the exponential increase in obesity worldwide. Early diagnosis is important in order to employ lifestyle and risk factor modification, and the research of novel natural remedies for prevention and treatment of MetS is the basis of reduction of standard therapy side effects.

In the present thesis, I focused the attention on omega-3 and omega-6 fatty acids, milk-derived peptides and abscisic acid, three compounds potentially involved in the regulation of plasma triglycerides, intestinal permeability and glycaemia. Three nutraceutical sources have been considered, *Salvia hispanica L.*, Buffalo Mozzarella cheese and peschiole, as a novel complementary and/or alternative safe remedy with clinical relevance in the MetS prevention.

The first aim was to test a chia seed-based nutraceutical formulation (CSN) for its potential effects on plasma triglyceride levels of healthy subjects with moderate dyslipidaemia. A cohort of 52 individuals were administered daily, for 8 weeks, with four gastro-resistant capsules of CSN, each one containing 500 mg of cryo-micronized chia seeds and 15 mg of vitamin E, according to a single centre, randomised, placebo controlled, 16 weeks trial. Data showed the following mean lipid changes: triglycerides, −27.5% (P = .0095); total cholesterol, −8.0% (P = .0019); High Density Lipoprotein cholesterol, +5.7% (P = .0042); Low Density Lipoprotein cholesterol, −10.2% (P = .0021).

Secondly, the bioactive properties of milk and milk-products are largely attributed to the peptides released during gastrointestinal digestion. Nevertheless, no similar studies on “Mozzarella di Bufala Campana DOP” (MBC), the European name given to a unique protected origin designation buffalo milk product, are available so far. A novel antioxidant peptide (MBCP) after MBC gastrointestinal digestion was identified and its *in vitro* intestinal protection, bioavailability, and anti-haemolytic capacity were assayed. A 0.2 mg/ml MBCP incubation dose made H_2O_2-stressed CaCo2 cell line proliferation increase by about 100%. Less than 10% hydrolysis in the apical solution and about 10% concentration in the basolateral solution indicated for MBCP good stability and

bioavailability, respectively. A 0.2 mg/ml MBCP incubation dose reduced H_2O_2-induced human erythrocyte haemolysis by 91.25%. The next step was to evaluate the therapeutic potential of MBCP in inflammatory bowel disease (IBD). I studied the effect of MBCP on (i) inflamed human intestinal Caco2 cells, (ii) dinitrobenzene sulfonic acid (DNBS) mice model of colitis and (iii) the administration in a clinical trial of a nutraceutical formulation corresponding quali-quantitative to MBCP composition. I have shown that MBCP, at non-cytotoxic concentrations, both *in vitro* and *in vivo* induced the adherens epithelial junctions organization, modulated the nuclear factor (NF)-κB pathway and reduced the intestinal permeability. Furthermore, the clinical trial showed, after 8 weeks of treatment, a reduction of lactulose/mannitol ratio, an index on intestinal permeability, of -75.2% ($P < .01$). The results obtained underline that MBCP possesses anti-inflammatory effects both *in vitro* and *in vivo*, and it is confirmed by a randomised, placebo controlled, study.

Lastly, I tested the effect of a peschiole-based novel nutraceutical formulation, rich in abscisic acid, on the metabolic parameters that are dysregulated in prediabetes and MetS. A cohort of 20 patients were administered daily, for 12 weeks, with two sachets for meal (breakfast, lunch, and dinner) corresponding to 30 µg abscisic acid. Data showed the following metabolic parameters mean: glycaemia, -26,85% *; insulin, -33,63% *; HOMA-IR, -58,89% *; glycated haemoglobin (HbA1c), -20,18% * (*$P < .01$).

Thus, CSN showed a clinical relevance in the primary cardiovascular disease prevention, MBCP helps to restore the intestinal epithelium integrity damaged by inflammation, and peschiole-based novel nutraceutical formulation may control plasma glycaemia, collaborating in the development of nutraceuticals useful for the treatment and prevention of MetS.

Introduction

Functional foods, according to The Institute of Medicine of the US National Academy of Sciences, are "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Menrad 2003). They are similar in appearance to a conventional food (beverage, food matrix), but contain biologically active components with demonstrated physiological benefits, and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions (Food and Agricultural Organization of the United Nations (FAO), 2007). Functional foods are often enriched or fortified with bioactive substances through a process called nutrification, examples should be food products that contain specific minerals, vitamins, fatty acids, dietary fibre, phytochemicals or antioxidants and probiotics. Some important examples of functional foods are represented by enriched cereals, breads, sport drinks, fortified snack foods, baby foods, prepared meals and more.

Hippocrates said, “Let food be thy medicine, and medicine be thy food”, suggesting that the ancients were aware of the importance of food for health. The concept of food as medicine is found also in folk sayings, such as “An apple a day keeps the doctor away” or “Good wine makes good blood”, which suggest that a certain type of food or beverage could prevent some kinds of disease.

Currently, there are many data that allow stating the importance of the combination between good nutrition and good health. For example, epidemiological and clinical trials have demonstrated that a nutrition rich in fish is associated with a minor risk of emergence of cardiovascular diseases, due to the effects of omega-3 fatty acids, such as EPA (eicosapentaenoic acid, C20:5 n-3) and DHA (docosahexaenoic acid, C22:6 n-3) (Kris-Etherton, et al. 2002). Other epidemiological trials have reported the existence of anticancer properties in green tea, due to its content in phenolic compounds, such as (-)-epigallocatechin gallate (EGCG) (Jankun, et al. 1997).

The specific bioactive compounds deriving from functional foods may be concentrate or subjected to an extraction process, producing the nutraceuticals. The word “nutraceutical” was coined in 1989 by Stephen DeFelice, founder and chairman of the Foundation of Innovation in Medicine (FIM), and it derived from the crasis of the term “nutrition” and “pharmaceutical”. A nutraceutical can be defined as “a food or a part of a food that provides medical or health benefits, including the prevention and/or the treatment of a disease” (Kalra 2003).

They may be used with the purpose of enhancing health, as a support therapy to conventional therapies in order to reduce dosages and side effects, and as a supplement to the diet of people with deficiency of some nutrients. Their dosages exceed those that could be obtained from normal foods (Zeisel 1999).

MetS is a cluster of metabolic diseases that includes hypertension, central obesity, insulin resistance, and atherogenic dyslipidaemia, strongly associated with an increased risk of developing atherosclerotic cardiovascular disease (CVD). Nowadays, it becomes particularly relevant because of the exponential increase in obesity worldwide (Rochlani, et al. 2017).

Existing therapies to treat the various pathological aspects of MetS are limited by the chronic nature of the diseases of MetS. Therapies, indeed, consist in a prolonged and often indefinite use of different medications, such as statins, leading to an increased burden of drug-related adverse effects and patient noncompliance. In this context, the development of nutraceuticals could contribute to decrease the dosage of MetS therapy and so to reduce side effects, or they could prevent or delay the onset of the disease.

Particularly, my attention has been addressed to three different food-matrices, *Salvia Hispanica L.* seeds (chia seeds), buffalo mozzarella cheese PDO (Protected Designations of Origin) and peschiole (green peaches). The former because represents a prominent vegetal source of alpha linolenic acid (18:3-ω3), the main polyunsaturated fatty acid involved in metabolism and physiological production of omega-3 fatty acids, an important hypotriglyceridaemic agents (Valdivia-López and Tecante 2015). Considering that dairy products and their derivatives milk bioactive peptides has been investigated for long time, and nowadays we are able to ascribe to them different biological activities (Mohanty, et al. 2016), I choose Buffalo mozzarella cheese to investigate the presence of bioactive peptides involved in intestinal inflammatory diseases and obesity.

Finally, peschiole are an agro-food industry waste deriving from fruit thinning. Their peculiar characteristic is the presence of abscisic acid, a phytohormone involved in the regulation of homeostasis glycaemic (Bruzzone, et al. 2012).

Thus, the aim of my thesis was to formulate and characterise novel nutraceutical products based on chia seeds, buffalo mozzarella peptides and peschiole, involved in regulations of the main factors of MetS, and to test them through *in vitro*, *in vivo* and clinical studies.

1 The metabolic syndrome

Metabolic syndrome (MetS) is a common metabolic disorder characterized by a cluster of components, among which: dysregulated glucose homeostasis, dyslipidaemia, hypertension, insulin resistance (IR) and central obesity. The presence of different above-mentioned factors is associated with increased cardiovascular diseases (CVD) and diabetes mellitus type 2 (T2DM). A pro-inflammatory (high TNF-α, interleukins, C-reactive protein) and pro-thrombotic state (high plasminogen activator inhibitor PAI-1) could contribute to the development of MetS.

Even though the concept of MetS exist since 1920, including hypertension, hyperglycaemia and gout, only 20 years ago was founded the term Metabolic Syndrome, considering that a striking increase in the number of people with the metabolic syndrome worldwide has taken place. This increase is associated with the global epidemic of obesity and diabetes, indeed the World Health Organization (WHO) established that one-third of adults over 20 years are overweight or obese. Nowadays there are conflicting opinions about the definitions of MetS as a pathology, or as surrogate of combined risk factors that put the individual at particular risk, or as a specific syndrome.

1.1 Definitions and diagnostic criteria

Reaven, a professor of medicine at Stanford University, in 1988 was the first to sustain a link between IR and the above-mentioned metabolic anomalies. He first named the syndrome "X", to then be renamed "MetS". Since then, different international organizations, like the WHO, the European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program Adult Treatment Panel III (NCEP: ATPIII), the International Diabetes Federation (IDF), and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), have tried to exactly define the criteria for the diagnosis of MetS.

In 1998, the WHO defined for the first time the criteria of MetS, underline the central role of glucose intolerance and IR as essential role for diagnosis, plus at least two further alterations including: dyslipidaemia, arterial hypertension, central obesity (as measured by waist/hip ratio or body mass index (BMI) and microalbuminuria.

One year later, the EGIR suggested to exclude T2DM and microalbuminuria in the criteria necessary to define the syndrome, while obesity was assessed by the waist circumference instead of BMI. Also, the EGIR definition requires two additional criteria selected from

obesity, hypertension and dyslipidaemia. Nonetheless, WHO and EGIR definitions agreed on the central role of IR in the pathophysiology of the syndrome.

In 2001, the NCEP: ATPIII published a new set of criteria better suited for clinical practice. MetS is diagnosed if at least three alternations of the following five criteria are present: waist circumference ≥ 102 cm (men) or ≥ 88 cm (women), triglycerides (TGs) > 150 mg/dl, high-density-lipoprotein cholesterol (HDL-C) < 40 mg/dl (men) or < 50 mg/dl (women), blood pressure > 130 mmHg systolic or > 85 mmHg diastolic, and fasting plasma glucose ≥ 110 mg/dl. The main difference between the NCEP: ATPIII and WHO and EGIR definitions is the absence of IR as main diagnostic parameter.

These parameters were slightly modified in 2004 by a group of experts from the AHA and NHBLI which have decreed a lowering of the hyperglycaemia threshold from 110 mg/dl to 100 mg/dl.

The later IDF criteria agreed with the NCEP: ATPIII definitions, except for central obesity as a requirement, measured by waist circumference or BMI ≥ 30 plus two other alterations must be present: dysglycaemia, low HDL levels, high TGs and/or hypertension.

Given the numerous controversies, there was a need to unify the criteria to diagnose MetS. Thus, in 2009, the IDF, AHA/NHLBI, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity, gathered and harmonised the definitions. Obesity was removed, while hypertension, hyperglycaemia and dyslipidaemia values were the same of NCEP definition. Moreover, waist circumference is country and population specific, thus, the harmonised criteria determine also the waist circumference cut-offs for different ethnicity.

1.2 Morbidities

Different studies demonstrated the correlation between MetS and the increased risk of developing CVD and T2DM. High levels of low-density-lipoprotein (LDL) and TG and decreased level of HDL-C are individually connected to cardiovascular risk, while IR increases the risk of developing T2DM. At last, different studies correlated central obesity with a higher incidence of CVD and T2DM.

In the Framingham Heart Study Offspring Study, patients with MetS had a relative risk of CVD of 2.88 for man and 2.25 for woman.

The risk of developing CVD and T2DM is strictly connected to the presence of additional risk factors which lead to diagnosis of MetS. People with isolated obesity have a T2DM developing risk significantly lower than those who presented MetS, or subjects with

isolated hypertension or dyslipidaemia have a lower cardiovascular risk than subjects who present multiple criteria. For example, in WOSCOPS (West of Scotland Coronary Prevention Study) the hazard ratio for T2DM was 7.3 with 3 parameters and 24.4 with 4 or more (Sattar, et al. 2003), instead the DECODE study showed a HR of CVD of 2.24 for males and 2.32 for females with abdominal obesity and 2 or more other components (Gao, et al. 2008).

Thus, the diagnosis of MetS can induce a 2-fold increase in CVD over 5 to 10 years and at least a 5-fold increase in development of diabetes.

1.3 Pathophysiology: IR, obesity, dyslipidaemia and hypertension

MetS is principally based on IR and obesity, two factor that, as we will see, are strictly linked.

Physiologically, insulin release from pancreas β-cell is stimulated by high plasma glucose, as it happens after meals. Consequently, insulin increases the uptake of glucose in muscle and adipose tissue stimulating the expression of Glucose transporter type 4 (GLUT-4) receptor and it inhibits hepatic gluconeogenesis and lipolysis. The result is a decrease in plasma glucose.

When the IR occur, liver, muscle and adipose tissue does not respond to insulin anymore and there is a condition of hyperglycaemia.

In visceral adipose tissue, in presence of IR, there is no response to insulin-mediated lipolysis, leading to a release of free fatty acids (FFAs) from triacylglycerol-rich lipoproteins. FFAs inhibit insulin-mediated glucose uptake in muscle, while promote gluconeogenesis and lipogenesis in liver. FFAs are also lipotoxic to β-cells, resulting in a decrease of insulin secretion. All these mechanisms contributing to hyperglycaemia.

Physiologically, insulin reduce ApoB production through PI3K-dependent pathways and promotes the activity of lipoprotein lipase, a protein involved in very-low-density lipoprotein (VLDL) clearance. The visceral fat increase in obesity lead to a massive lipolysis, causing a higher flow of FFAs to liver. Here, they serve as substrate in TG synthesis and contribute to production of ApoB, the main lipoprotein of VLDL. They are rapidly metabolised in LDL, which contribute to development of atheroma. Moreover, TG in VLDL are transferred to HDL, forming TG-enriched HDL, a better substrate for hepatic lipase. In this way HDL is removed from bloodstream, resulting in a minor reverse cholesterol transport, the reason why low HDL value is one of the main diagnosis criteria

of MetS. Thus, insulin resistance leads to hypertriglyceridemia (HTG), increase of LDL and reduction of HDL.

The endocrine function of adipose tissue contributes to MetS development. Adipocytes, physiologically, secrete differ adipokines, such as leptin, resistin, retinol, visfatin, plasminogen activator factor 1, which induce IR. In obesity, high level of leptin is observed, resulting in hyper-activation of immune cells and increase in CVD risk.

On the contrary, adiponectin has an anti-inflammatory and anti-atherogenic effect, reduce the vascular reactivity and stabilise the atherogenic plaque, hence it is considered a protective factor for diabetes and hypertension (Gao, et al. 2008). The unbalanced ratio of high leptin and low adiponectin in obesity induce CVD diseases risk.

It has been demonstrated the increase of macrophages in adipose tissue of obese individual (Weisberg, et al. 2003). They could be responsible to improve multifactorial inflammation state of MetS because of the secretion of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Their production increase with increase of body fat and they are correlated to IR. TNF-α promote the phosphorylation, and so inactivation, of insulin receptor follows by a decrease in the activation of phosphatidylinositol-3-kinase, the second messenger that regulates most of the metabolic effects of insulin (Hotamisligil, et al. 1994). Another mechanism could be the induction of hepatic lipogenesis and the inhibition of adiponectin release (Kern, et al. 2003). IL-6, instead, has an endocrine function. Secreted by adipose tissue, it stimulate hepatic TG secretion and gluconeogenesis, causing an alteration of insulin secretion (Fasshauer and Paschke 2003). IL-6 it is also involved in increased production of C-reactive protein (CRP), the most important marker of metabolic risk because it is involved in vascular damage (Burke, et al. 2002). Several studies sustained the correlation between high CRP and MetS, diabetes and CVD. At last, IL-6 increase fibrinogen activation, increasing platelet and thrombotic activity (Burstein, et al. 1996).

Lastly, development of hypertension is multifactorial: 1) FFAs and angiotensin II, produced by adipocytes, leading to production of reactive oxygen species (ROS), which contribute to LDL oxidation and endothelial dysfunction (Bergman, et al. 2007); 2) IR leads to altered signalling of vasodilatation NO-dependent and vasoconstriction endothelin-1 dependent, resulting in increase of cellular proliferation and adhesion molecules and vasoconstriction determined by reduced production of Nitric Oxide (Kim, et al. 2006); 3) hyperactivity of renin-angiotensin-aldosterone system (Landsberg, et al. 2013); 4) overabundance of cytokines produced by adipose tissue.

1.4 Therapy

The primary outcome in MetS therapy is the treatment of individual components or reduce the CVD and T2DM risks. According to NCEP: ATPIII, there are 2 major therapeutic goals: lifestyle modifications, leads to weight loss and increase of physical activity, and treatment of CVD risk factors. As regards lifestyle modifications, weight loss has been proven useful for MetS treatment (Lloyd-Jones, et al. 2007). According to guidelines, the goal is a weight loss of 7-10% by one year, in order to achieve BMI < 25 kg/m^2 (Grundy, et al. 2004). The Diabetes Prevention Program (DPP) trial, proved a 41% reduction of incidence of MetS after 3 years of intensive lifestyle modifications (Orchard, et al. 2005). Dietary recommendations were published in an update of NCEP criteria. They included: a diet low in simple sugar (< 25% of caloric intake), reduce total fat to 25% of calories, reduce saturated FA and increase unsaturated FA, reduce cholesterol intake and a daily sodium intake between 1,5 and 2 g/day, in order to decrease blood pressure (Appel, et al. 2006; Maron, et al. 1991)

Lifestyle modification included also physical activity that play an important role in reduction of visceral fat and enhancing insulin sensitivity. American Heart Association and American College of Sports Medicine recommend moderate aerobic activity for at least 30 min/day for 5 days a week (Haskell, et al. 2007). When weight loss and physical exercise are inadequate to reach clinical benefit, pharmacologic therapy is used to treat cardiovascular risk. Patients with BMI ≥ 30 kg/m^2, or ≥ 27 kg/m^2 in presence of risk factors, can be treated with orlistat, an anti-obesity drug working as inhibitor of gastric and pancreatic lipase (Torgerson, et al. 2004). IR and hyperglycaemia needs a treatment with metformin when both impaired fasting glucose and impaired glucose tolerance occurred, namely when diabetes is diagnosed (Nathan, et al. 2006).

Patients with MetS have, generally, high level of TGs, low HDL-C and consequently an increase in small dense LDL value. The primary target, according to NCEP: ATPIII criteria, is reach LDL-C value < 100 mg/dL in high-risk patients, using statins. Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are the elective drug for treatment of hypercholesterolemia, allowing lowering of LDL-C of 15% to 60%, and can be used alone or in combination with cholesterol absorption inhibitor (ezetimibe) or bile acid sequestrants (colestipol, cholestyramine). Used alone, the first reduce of 15-25% LDL-C, the latter of 15-30%.

The secondary target of dyslipidaemia therapy consists in increase HDL levels and decrease TGs if they are > 500 mg/dl. Niacin is the FDA-approved drug for raising HDL-C

(15%–35%), lowers TGs (20%–50%) and LDL-C (5%–25%). Nonetheless, two recent clinical trials showed no significant improvement in HDL levels in niacin treated group compared to patients already in treatment with statins (Group 2013; Investigators 2011)
On the other hand, fibrates reduce TGs from 25 to 50% and increase HDL-C from 5 to 15%. Fibrate therapy with gemfibrozil demonstrated a reduction in incidence of coronary heart disease and a higher reduction of HDL-C in combination with statin in high risk patients, even if their association cause myopathy (Rubins, et al. 1999). Moreover, the association fibrates-statins in patients with T2DM, showed good results as regards TGs and HDL-C reduction (Ginsberg, et al. 2007).
Finally, pharmacological treatment of hypertension should start when blood pressure is ≥140/90 mmHg, otherwise lifestyle modification and reduction of sodium intake are enough. The elective drugs to treat hypertension in MetS are angiotensin-converting enzyme (ACE) inhibitor and angiotensin receptor blockers, because of hyperactivity of renin-angiotensin-aldosterone system in obesity (Landsberg, et al. 2013). Thiazide-type diuretics and β-blockers, instead, are not recommend because of alteration of metabolic profile and increasing in IR, respectively (Landsberg, et al. 2013).
Thus, there is not a single drug for MetS therapy, but the co-occurrence of pathologies needs a prolonged polypharmacy which cause a reduced compliance for patients. Moreover, drug side effects are well known. I.e. niacin cause hyperglycaemia, skin flushing and uric acid elevations; statins cause myalgia; metformin cause diarrhoea, lactic acidosis and flatulence; ACE inhibitors cause cough; fibrates cause gastrointestinal disorders and bile acid sequestrant cause constipation and bloating. Hence, alternative, natural, and safer remedies are still in great demand.

2 Hypertriglyceridemia

HTG refers to an increased fasting plasma TGs measurement. According to the Endocrine Society clinical practice and the NCEP: ATPIII guidelines (Berglund, et al. 2012; Detection and Adults 2002), physiologically value of blood TGs are < 150 mg/dl. Classification of HTG can be observed in Table 1.

NCEP: ATPIII		The Endocrine Society	
Borderline-high TG	150–199 mg/dl	Mild hypertriglyceridemia	150–199 mg/dl
High TG	150–199 mg/dl	Moderate hypertriglyceridemia	200–999 mg/dl
Very high TG	≥500 mg/dl	Severe hypertriglyceridemia	1000–1999 mg/dl
		Very severe hypertriglyceridemia	≥ 2000 mg/dl

Table 1 *Definition of hypertriglyceridemia*

Abbreviations: mg/dl, milligram/decilitre; TG, triglycerides

TGs are lipids composed by three chains of fatty acids and one molecule of glycerol. They are hydrophobic molecules stored in adipose tissue, which represent an important source of energy and can be transported between tissues through lipoproteins.

Dietary TGs are absorbed in the gut and incorporate in chylomicrons, while endogenous TGs are incorporated in VLDL particles, the main TG carrier produced by the liver.

TG content of chylomicrons is rapidly hydrolysed by lipoprotein lipase (LPL) on capillary endothelial cells of adipose and muscle tissues, releasing free fatty acids. This process leads to the formation of chylomicron remnant, a molecule enriched in cholesteryl esters, retinyl esters and apo B-48 (Berglund, et al. 2012).

VLDL TGs are similarly hydrolysed by LPL, leaving VLDL remnants in the bloodstream and intermediate-density lipoprotein (IDL), a small particles enriched in cholesteryl esters (Feingold and Grunfeld 2018).

In patients with HTG, increased levels of TG in the bloodstream are correlated to the activation of cholesteryl ester transfer protein (CETP) and LPL. The former transfer TG from IDL and VLDL into HDL and LDL particles, in exchange for cholesteryl esters (Guérin, et al. 2001). This leads to an increase in TG content of HDL and LDL. Thus, TG-enriched HDL are rapidly metabolised and have a short plasma half-life, while TG-enriched LDL are hydrolysed by LPL, generating small dense LDL particles, with a high atherogenic potential.

HDL reduction and small dense LDL increase is associated with an increase in TG-rich lipoproteins or remnant lipoprotein particles (RLP), that, as well as small dense LDL, have a pro-atherogenic activity. Indeed, it has been showed that lipolysis of TG-rich lipoproteins on the surface of endothelial cells by LPL, yields a cholesterol-rich remnant (RLP). RLP contain five to twenty times more cholesterol than LDL and can cross the endothelial barrier, where they are internalise by macrophages and concur in the foam cell deposition, the hallmark cells of the atherosclerotic plaque (Varbo, et al. 2013).

Moreover, apolipoprotein C3, a glycoprotein located on TG-rich lipoproteins, also concur in atherosclerosis development because inhibits LPL and exhibits pro-inflammatory activities (Ooi, et al. 2008).

2.1 Classification

HTG may be primary or secondary, depending on the presence of genetic component (Yuan, et al. 2007).

The primary one is a result of some genetic defects that leads to impaired TGs metabolism, as shown in Table 2. The secondary is related to high-fat diet, obesity, T2DM, hypothyroidism, alcohol abuse, renal diseases, pregnancy, and medications (corticosteroids, oral oestrogens, tamoxifen, isotretinoin, beta blockers, thiazides, antipsychotics and bile acid resins).

Primary hypertriglyceridemia	
Name	*Characteristics*
Familial chylomicronaemia	Presence of chylomicrons after 12-14 hours period of fasting LPL and apoC2 deficiency
Familial hypertriglyceridemia	Polygenic: high TG due to excess hepatic VLDL production and normal cholesterol levels
Familial combined hyperlipoproteinemia	Autosomal dominant (polymorphisms in molecules and enzymes participating in lipoprotein metabolism (apoC2, apoC3) Increased VLDL and LDL, depressed HDL
Familial dysbetalipoproteinaemia	Homozygosis for APOE E2 isoform Increased IDL, total cholesterol and TG

Table 2 *Classification of primary hypertriglyceridemia*

Abbreviations: LPL, lipoprotein lipase; apo, apolipoprotein.

2.1.1 *Consequences*

HTG is a risk factor for pancreatitis and represents 1 to 4% of cases of acute pancreatitis. Though some patients may develop pancreatitis with TG levels > 500 mg/dl, the risk of pancreatitis becomes clinically significant for TG levels > 1000 mg/dl. HTG, from a clinical point of view, is frequently associated with other lipid abnormalities and MetS, widely known risk factors for coronary heart disease.

TG values > 500 mg/dl may be the cause of eruptive xanthomas or lipemia retinalis. In some cases, extremely high levels of chylomicrons can cause the Chylomicronaemia syndrome, characterised by recurrent abdominal colic, nausea, vomit and pancreatitis: usually in this condition TGs levels are > 2000 mg/dl.

2.1.2 *Treatment*

In case of mild-moderate HTG (TG < 200 mg/dl), US and European guidelines suggest, as first line treatment, lifestyle modifications such as limit alcohol assumption, fat and sugars intake, weight loss and regular moderate-intensity exercise program (Catapano, et al. 2016; Miller, et al. 2011). Following the Mediterranean diet is also suggested because of the high content of monounsaturated FA, able to reduce TG and increase HDL values (Kastorini, et al. 2011). These precautions lead to TG reduction of 20-30%.

If lifestyle modifications are not enough to decrease TG levels or in case of severe HTG (TG > 200 mg/dL), it is required a pharmacological treatment, including fibrates, nicotinic acid, statins or ω-3 FA (Catapano, et al. 2016; Siscovick, et al. 2017).

Statins are the first choice to reduce both the total risk of CVD and moderately elevated TGs (Topol 2004). Potent statins, such as atorvastatin and rosuvastatin, are more effective in reducing TGs, especially at high doses and in patients with high triglyceridemic levels.

Fibrates are peroxisome proliferator-activated receptor-α agonists, a transcription factor that regulate different phases of lipid and lipoprotein metabolism. In particular, they reduce hepatic VLDL secretion and increase lipolysis of plasma TGs (Rubins, et al. 2002). Fibrate therapy may reduce major cardiovascular events by 13%, particularly in patients with high TGs levels (> 200 mg/dl). The main side effect is myopathy. The risk of myopathy has been reported to be 5.5 times greater when fibrates are used in monotherapy than in association with statins, and it change according to the type of fibrate and statin used. These risks are related to the interaction between gemfibrozil and statins glucuronation, increasing plasma concentrations of statins. Since fenofibrate has a different pharmacokinetics than gemfibrozil, fenofibrate-statin combination therapy is much less risky for the onset of myopathy (Jones and Davidson 2005).

Nicotinic acid decreases the flow of fatty acids to the liver and hepatic secretion of VLDL, as well as IDL and LDL. Moreover, it increases HDL cholesterol, mainly stimulating the hepatic production of apo-A1. At a dosage of 2 g/day is observed a reduction of TGs of about 20-40% and LDL-C by 15-18%, and an HDL-C increase of 15-35% (Carlson 2005). The main side effects are skin reactions (redness) and a slight increase of glycaemia.
Finally, ω-3 fatty acids are used to reduce TGs levels because they influence serum lipids and lipoproteins, in particular LDL. The recommended doses of EPA and DHA for reduce TGs vary between 2 and 4 g/day. The FDA has approved the use of Omega-3 fatty acids in addition to diet modification, if TGs exceeds 500 mg/dl (Lee, et al. 2008). Their administration appears to be safe and free of clinically significant interactions, although their anti-aggregating action limit the co-administration with aspirin/clopidogrel.

2.2 Polyunsaturated fatty acids: omega-3 and omega-6

Omega-3 (n-3) and omega-6 (n-6) belongs to the family of polyunsaturated fatty acids (PUFAs). Their name is due to the presence of a methyl end, also known as omega end, and a carboxylic acid group in the opposite side. The name omega-3 (ω-3) and omega-6 (ω-6) means the presence of the first desaturation three and six carbon, respectively, after the omega end. Plant can synthesise linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3), while animals and humans not. So, these fatty acids are known as essential because they must be introduced with diet. Dietary source of ω-3 PUFA, especially EPA and DHA, are seafood like tuna, salmon, sardines and mackerel, while plants and vegetable oils are rich in ω-6 linoleic acid (Dawson, et al. 2014). Once in the body, thanks to the action of Δ5- and Δ6-desaturase and different elongase, α-linolenic acid is converted into eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA), while linoleic acid for the most part is converted in arachidonic acid (AA) (Widmer and Holman 1950).

2.2.1 Anti-inflammatory properties

The ω-6 fatty acid AA, in most cell type, is the precursor of eicosanoids, important regulators and mediators of inflammatory processes. Indeed, AA is stored within cellular membrane and can be converted, through the action of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450, into prostaglandins, leukotrienes and thromboxane. Thus, a diet rich in ω-6 is responsible of inflammatory, pro-constrictive and prothrombotic state, even because AA derived eicosanoids are active in low quantities. Supplementation of EPA and DHA increase their relative abundance in membrane cell

phospholipids, where they replace and so reduce the storage of AA, resulting in a lower production of inflammatory eicosanoids. Moreover, EPA and DHA are subjected to the same metabolism of AA, with the result of leukotrienes and prostaglandins with anti-inflammatory properties. Is has been shown that EPA and DHA derived eicosanoids are structural and functional distinct from those originating from AA, besides being less powerful as inflammatory compounds (Lands 1992).

Mechanism wherewith ω-3 PUFA are involved in regulation of inflammation are different. First, they affect the production of cytokines and adhesion molecules. Is has been showed that EPA and DHA administration, significantly decrease TNF-α, IL-6 and IL-1 β secretions in response to lipopolysaccharides (Calder 2015). In particular, EPA is much more effective than DHA on modulation of TNF-α, whereas DHA is more potent on reduction of IL-6 (Mullen, et al. 2010). Secondly, they decrease expression of adhesion molecules like vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 in monocytes and macrophages, while they inhibited nuclear factor-kB (NF-kB) activation, a pro-inflammatory transcription factor, resulting in modulation of adipokines levels (Siriwardhana, et al. 2012).

Finally, EPA and DHA are involved in production of pro-resolving lipid mediators, resolvins, protectins and maresins, synthesised from COX-2 and LOX in the presence and absence of aspirin. Resolvins are produced both from EPA (E-series) and DHA (D-series), while protectins and maresins derived only from DHA. Different *in vitro* and *in vivo* studies demonstrated their anti-inflammatory and pro-resolving properties. For example, resolvin E1, resolvin D1 and protectin D1 all inhibit trans-endothelial migration of neutrophils into sites of inflammation; resolvin D1 inhibits IL-1β production and protectin D1 inhibits TNF-α and IL-1β production (Bannenberg and Serhan 2010; Serhan and Chiang 2013; Serhan, et al. 2008).

Thus, EPA and DHA dietary ingestion can altered the content of AA and the ω-3/ω-6 ratio (Simopoulos 2008) became a main factor to modulate the inflammation processes.

2.2.2 Effects on CVD and coronary heart disease mortality

In 1970, Bang and Dyerberg studied the Greenland Inuit diet and they observed a lower risk of developing CVD and atherosclerosis compared to other population, due to high-fish consumption (Bang, et al. 1980). ω-3 PUFA were found to be responsible of this effect (Fodor, et al. 2014) and, since then, numerous studies monitored the effect of PUFA on CVD in order to demonstrate a correlation.

One of the earliest study evaluating the effect of diet on CVD was the Oslo study, where healthy men at high risk of coronary heart disease (CHD) were subjected to a diet aimed to low cholesterol and they were encouraged to quit smoking (Hjermann, et al. 1981). After 5 years of observation period, there was a 47% of reduction of total coronary events compared to control.
The Diet and Reinfarction Trial (DART) study enrolled 2033 men recovering from myocardial infarction (MI). The group advised to eat from 200 to 400 g of fatty fish weekly, equivalent to 500 to 800 mg of ω-3 PUFA daily, showed a 29% reduction of all-cause mortality and a 16% reduction of ischemic events after 2-years (Burr, et al. 1989).
Although the benefit outcome of this studies depends on population, a recent meta-analysis of 32 cohort studies on self-reported dietary fatty acid intake found a reduction of CHD risk in patients of the top tertile compared to the lowest one (Chowdhury, et al. 2014). Other meta-analysis showed that the ingestion of fish or fish oil reduce CHD mortality in patients with and without CVD (Leon, et al. 2008; Marik and Varon 2009)
Dietary intervention trial was followed by interventional outcomes studies to ascertain the effect of ω-3 PUFA on lowering CVD risks. The Gruppo Italiano per lo studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione trial studied the effect of administration of ω-3 supplements, containing 850 mg of EPA/DHA in ratio 1.2:1, with and without vitamin E, on 11323 patients with a history of MI. Patients in treatment with only ω-3 showed a reduction of 30% for cardiovascular death and of 45% for sudden death (Valagussa, et al. 1999).
Other interventional outcomes studies conducted did not showed a statistically significant reduction of CHD mortality compared to controls (Blacher, et al. 2013; Bosch, et al. 2012; Kromhout, et al. 2010; Rauch, et al. 2010) after myocardial infarction, even because they had different limitations, such as interrupted recruitment after 7 years, no evaluation of medications changing or eating advice to the control group based on healthy diet.
The association statins and EPA was observed in JELIS, demonstrating a reduction of cardiovascular risk (Yokoyama, et al. 2007), while 9 international outcome studies enrolled patients with HTG (mean or median baseline TG levels ranging from 97–163 mg/dL) showed that ω-3 FA significantly reduce TG levels (Bosch, et al. 2012; Einvik, et al. 2010; Tavazzi, et al. 2008).
Thus, the conflicting results observed in international outcomes underline that the effects of ω-3 FA on CVD and CHD were not evaluated adequately, especially for study

limitations. The most promising benefit are expected to the association of PUFA and statins and hyper-triglyceridemic subjects.

2.2.3 Effects on arrhythmias

Different *in vitro* studies demonstrate the effect of ω-3 FA on onset of atrial fibrillation (AF), due to favourable changes in left ventricular performance, autonomic tone, or inflammation. Clinical trial showed different controversies about this effect. The Diet, Cancer, and Health Cohort Study enrolled 57053 Danish patients, subjecting them a food questionnaire on fish ω-3 intake. There was observed an U-shaped association between consumption of fish and ω-3 FA (EPA and DHA) and risk of incident AF, with the lowest risk of AF at a moderate intake of 0.63 g/day (Rix, et al. 2014).

Instead, a recent study of Darghosian, et al. (2015) showed no significance correlation between ω-3 FA and a reduced recurrence of AF in patients who take 4g/day of ω-3 PUFAs. Moreover, six small randomised clinical trial have evaluated the effect of PUFA supplementation on postoperative AF after cardiac surgery (Calo, et al. 2005; Farquharson, et al. 2011; Heidarsdottir, et al. 2010; Heidt, et al. 2009; Rodrigo, et al. 2013; Saravanan, et al. 2010). Only three of the six trials found a positive correlation, with the varying designs and small sizes limiting strong conclusions.

2.2.4 Effects on hypertriglyceridemia

Several epidemiological study correlated high plasmatic TG levels and CVD risk (Cullen 2000; Freiberg, et al. 2008; Hokanson and Austin 1996; Labreuche, et al. 2009; Nordestgaard, et al. 2007), showing that every 88 mg/dl TG decrease reduces CHD risk of 14% in men and 37% in woman (Hokanson and Austin 1996), focusing the attention on treatment of HTG for prevention of CVD. Higher TG levels, indeed, are often present in subject with high cardiovascular risk, including those with dyslipidaemia, MetS and T2DM.

As aforementioned before, if lifestyle modifications are not enough to decrease TG levels or in case of severe HTG, it is required a pharmacological treatment, including fibrates, nicotinic acid or ω-3 FA (Catapano, et al. 2016; Siscovick, et al. 2017). The most used and effective fibrates therapy, give side effects includes high creatinine, myopathy and rhabdomyolysis, switching the attention on other therapies (Davidson, et al. 2007). Thus, ω-3 FA are an important alternative pharmacological therapy, alone or in combination with statins or other TG-lowering drugs in presence of severe HTG (Catapano, et al. 2016).

EPA and DHA combination from 2 to 4g/day decrease TG by 25-30%, and the most promising decrease is observed when baseline TG levels are higher (Harris, et al. 1997). The mechanisms whereby EPA and DHA act on TG levels are different (Karalis 2017): 1)downregulate hepatic VLDL synthesis (Shearer, et al. 2012); 2) increase β-oxidation of FA in liver, skeletal muscle, cardiac and adipose tissue (Shearer, et al. 2012); 3) increase FA uptake in adipocytes, skeletal and muscle cells ; 4) decrease post-prandial lipemia, from which derived atherogenic and thrombogenic lipoproteins (Harris, et al. 1988; Miyoshi, et al. 2014); 5) decrease TG content of chylomicrons and VLDL (Miyoshi, et al. 2014); 6) enhance clearance of plasmatic TG.

It has been shown also a potential beneficial effect on HDL particles, increasing HDL2/HDL3 ratio, where HDL2 is a large cholesterol rich subtype with antiatherogenic effect and HDL3 is tri-glycerol enriched one (Harris, et al. 1988; Mori, et al. 1999). ω-3 FA are also able to positively modulate HDL associated proteins, such as clusterin, Apo-AI, ApoCIII and ApoE, and so HDL functionality (Burillo, et al. 2012). In the meantime, we could assist in a slight increase in LDL-C due to increase conversion of VLDL to LDL, even if it is not significant compared to TG reduction. LDL-C increase does not involve the particles number, rather a switch from the smaller LDL to the larger one, that are much less atherogenic than the first one (Calabresi, et al. 2000). A recent meta-analysis attributed this effect only to DHA and not to EPA (Woodman, et al. 2003).

The main pharmaceutical preparation of EPA (47%) and DHA (38%) contains them as ethyl ester form. Patients with TG levels > 500 mg/dl were given 4g/day of the above cited preparation and, after 4 months, showed a TG reduction of 45%, VLDL 32% and an increased in HDL of 13% and LDL of 31% (Harris, et al. 1997). Nonetheless, ethyl esters form needs an enzymatic digestion prior to be absorbed in human body and it depends on fat meal content. Recently, a novel pharmaceutical form of EPA (55%) and DHA (20%) free fatty acids has been developed and it showed a better bioavailability of ethyl ester forms (Davidson, et al. 2012). This formulation was tested in the EVOLVE trial, where a dose of 4g/day showed a TG reduction of 31% (Kastelein, et al. 2014). MARINE trial, instead, test the formulation AMR101 which contains EPA for at least 96% and it showed a decrease in TG levels of 33.1%, after the assumption of 4g/day, while LDL-C values were unchanged, probably due to the absence of DHA (Bays, et al. 2011).

Finally, ω-3 FA have proved capable of decrease plasmatic TG in subject with high HTG and in patients at risk of CVD (Yokoyama, et al. 2007), while in patients with proven CVD risk the effect of ω-3 is less certain. Nonetheless, the lack of randomised controlled data

prompted the European Heart Association to recommend pharmacological treatment in patients with TG > 200 mg/dl and controlled LDL-C by using fibrates and only in case of contraindications use ω-3 FA (Catapano, et al. 2016).

2.3 Chia and chia seeds

Chia seeds are small, flat and oval seeds rich in properties (Fig.1). They are generally grey-black in colour and represent the seeds of *Salvia hispanica, L.*, a floral plant extremely common in central and southern America, especially in Mexico and Guatemala, where they represented the fundamental food of ancient populations such as Aztec and Maya. Indeed, they used chia seeds as a remedy against inflammations and infections and during battles because it was able to give strength (in Aztec "Chia" means "strength"). At that time, Chia seeds were an extremely important product in the agri-food sector as they were comparable to corn, beans and amaranth, at the base of the nutrition of civilizations.

Fig.1 *Raw Chia seed, on the left, and chia seed mucilage when soaked in water, on the right.*

Salvia hispanica is an herbaceous plant with leaves that vary from 4 to 8 cm in height and with a width of 3-5 cm, purple or white flowers and can reach about one meter in height. The seeds colour varies from black, grey, and black spotted to white, and the shape is oval with size ranging from 1 to 2 mm (Bresson, et al. 2009). Their composition is variable and depends on the region where they grows (Ayerza 1995).
The plant is still cultivated in the same territories above-mentioned, both to produce seeds and flour, but can be also cultivated in mild climates.
Chia seeds are very valuable because of their nutritional properties (Table 3), such as the high calcium content and the presence of essential fatty acids ω-3 and ω-6, comparable to

sesame and flax from a nutritional point of view. Nevertheless, chia seeds result much more valuable than flax seeds in terms of nutritional facts, specifically, as regards their higher amounts (for 100 g seeds) of calcium (631 mg vs 255 mg), fibre (34.4 mg vs 27.3 mg), phosphorus (860 mg vs 642 mg), their superior levels of antioxidants (mainly polyphenols) and proteins characterised by a high biological value (https://ndb.nal.usda.gov), and, of no secondary importance, their much lower contents in compounds of toxicological concern. Moreover, chia seeds are a good source of niacin and vitamins A and C.

Nutritional values for 100 g of Chia seeds	
Energy value	490 Kcal
Water	4,9 g
Carbohydrate	43,85 g
Fibre	37,7 g
Total fat	30,75 g
Saturated fat	3,176 g
Monounsaturated fatty acids	2,115 g
Polyunsaturated fatty acids	23,335 g
Protein	15,62 g
Cholesterol	0 mg
Calcium	631 mg
Phosphorus	948 mg
Manganese	2,167 mg
Potassium	160 mg
Copper	0,188 mg
Sodium	19 mg
Zinc	3,49 mg

Table 3 *Nutritional values of Chia seeds*

2.3.1 Properties of Chia seeds

2.3.1.1 High fibre content

Dietary fibre (DF) is a mixture of plant carbohydrates polymers, such as cellulose, pectin, gums and lignin, and noncarbohydrate molecules, such as polyphenols, saponins and waxes (Elleuch, et al. 2011).

Chia seeds, as showed in Table 3, contains about 37% of total dietary fibre (TDF), of which 6% of soluble DF and 32% of insoluble DF.

The right daily intake of fibre, according to the American Dietetic Association, is between 25 and 30 g/day for adults. Two tablespoons (about 10 g) of chia seeds correspond to more than one-third of the daily recommended fibre intake.

TDF exert different health beneficial effects on our body, such as increase of the sense of satiety, improving intestinal transit, reduction of cholesterol, modification of glucose and insulin-mediated response, antioxidant activity and reduction of diverticular disease risk (Crowe, et al. 2014).

Insoluble DF is the predominant fraction in chia seeds, of which the main component is Klason lignin. The latter protects unsaturated fats in chia seeds thanks to its resistant structure and antioxidant compounds (Reyes-Caudillo, et al. 2008), and reduce cholesterol values due to its capacity to absorb bile acids. Moreover, insoluble DF can be fermented in the intestine by promoting the formation of short chain fatty acids, thereby improving colon health.

Soluble DF, instead, is composed of neutral sugars able to form a mucilaginous structure when seeds are soaked in water. Indeed, they have a high hydration capacity and they can absorb up to 27 times their weight in water, forming a gelatinous mass around the seed, which helps to stimulate the intestinal peristalsis and give a greater sense of satiety. Nonetheless, it also provides a very low bioaccessibility of ω-3 FA (Goh, et al. 2016).

2.3.1.2 High ω-3 content

Chia seeds contains about 30% of total fat, highly unsaturated, with 60% of it comprising alpha-linolenic acid (ALA; 18:3 ω-3) and 20% of linoleic acid (18:2 ω-6). Both essential fatty acids are required by the human body for good health, and they cannot be artificially synthesised. In particular, seeds have the ω-3/ω-6 FA ratio of about 4:1 (Ciftci, et al. 2012). The amount of PUFA depends on the region of cultivation, rather than the colour of chia seed coat (Ayerza 2010).

The Institute of Medicine (IOM) of the National Academies established for ALA an Adequate Intake (AI) of 1.6 g/day for men and 1.1 g/day for women (Trumbo, et al. 2002). The IOM noted that intakes of ALA above the AI may confer additional health benefits, especially with respect to cardiovascular health. Many advisory boards consider ALA intakes greater than 1.5 g/day important for human health (Gebauer, et al. 2006).

Chia ω-3 FAs has different health effects, among which CVD risk reduction, TG levels decrease, HDL increase and it is involved in hypertension, allergies, endocrine disorders and cancer (Ulbricht, et al. 2009). Vuksan, et al. (2007) demonstrated that consumption of 37 g/day of Chia oily seed has a positive effect on cardiovascular risk factors by reducing inflammatory markers and blood pressure in T2DM patients.

2.3.1.3 *Proteins content*

Chia seed protein content is in average 16-23%, even in this case depending on the geographical area of plantation. Protein and amino acids content are both greater than other grains and cereals (Ayerza and Coates 2001).

Olivos-Lugo, et al. (2010) studied the amino acids profile, showing a high amount of glutamic acid (123 mg/g), arginine (80.6 mg/g) and aspartic acid (61.3 mg/g), all expressed as mg/g of raw protein.

Finally, it has been reported an emulsifying activity of protein-rich fraction obtained from *S. hispanica* seeds (Vázquez-Ovando, et al. 2013) and an ability to form elastic film with a very low permeability to oxygen, when glycerol or sorbitol are incorporated as plasticiser. This property is quite important in improve food quality, since oxygen it the most frequent cause of oxidation in food matrixes, causing colour, flavour and nutrients deterioration (Hernández-Jardón 2007).

2.3.1.4 *High polyphenolic content*

Chia seeds are rich in antioxidant, mainly represented by polyphenols. They may be present in free form or bonded to sugars by glycosidic linkages, which allow to increase their solubility in water.

It is widely reported that the most important polyphenolic compound of chia seeds are phenolic acids, such as chlorogenic and caffeic acids, and flavanols like quercetin, kaempferol and myricetin (Ayerza and Coates 2001). Furthermore, a more recent study demonstrated the presence in chia extract of rosmarinic, gallic and ferulic acids, and glycitin, genistin and genistein (Martínez-Cruz and Paredes-López 2014). Among them, rosmarinic acid is the most abundant (0.926 mg/g).

Thus, chia seed may be used as an anti-oxidative source since it is stronger than common antioxidant, such as vitamin C and E, and caffeic and chlorogenic acids inhibit lipid peroxidation.

2.3.1.5 *Chia as a functional food*

Chia can be classified as a "functional food" because its valuable contribute to increase satiety index, prevents cardiovascular and inflammatory diseases, and diabetes (Muñoz, et al. 2013).

Chia seeds, hence, can be eaten raw or can be used as oil supplement, and for preparation of bakery products, salads, cereals, cake, yogurt or fruit juices (Mohd Ali, et al. 2012). In 2000, the US Dietary Guidelines recommended a maximum intake of 48 g/day.

3 Intestinal permeability

The intestine is at the interface between the organism and its luminal environment, thus, representing a critical defence barrier against luminal toxic agents. Indeed, there are a particular intestinal epithelial cell, tight junctions (TJs), having a barrier function and involved in the regulation of paracellular permeability (Scaldaferri, et al. 2012). Therefore, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated ROS (Aw 1999). Thus, an alteration of intestinal mucosa, specifically the breakdown of TJs, is the basis of intestinal diseases mainly known as *leaky gut syndrome* (Michielan and D'Inca 2015).

Several studies (Liu, et al. 2005; Michielan and D'Inca 2015; Playford, et al. 2001; Scaldaferri, et al. 2012) have confirmed the role of leaky gut syndrome in many systemic and autoimmune diseases, such as ulcerative colitis, Crohn's disease, coeliac disease and type 1 diabetes mellitus (Lerner and Matthias 2015). The loss of protective function of mucosal barriers in association with a genetic predisposition are necessary to develop autoimmunity (Liu, et al. 2005).

Therefore, a method to prevent leaky gut syndrome would have a substantial clinical value. Currently, the most promising therapies based on natural substances and indicated for disorders associated with alterations of the IP, are represented by colostrum and milk derived proteins and peptides. These molecules influence cell growth, acting as growth factors on gastrointestinal tract, and preserve the epithelial barrier, acting directly on TJs (Prosser, et al. 2004). The most abundant growth factors in bovine milk and colostrum are epidermal growth factor (EGF) (Hollander 1999), insulin-like growth factors IGF-I and IGF-II (Lerner and Matthias 2015), transforming growth factors TGF-α (Playford, et al. 2001) and TGF-β (Drossman, et al. 2009). They are physiologically present in the intestinal lumen and the distribution of most of their receptors are on the basolateral side of membrane, so in healthy gut this receptor sites could be not reachable. Instead, in leaky gut syndrome, IBD and IBS, growth factor-binding receptors might undergo a shift from basolateral to apical membranes enhancing, thus, the ligand-receptor interaction, resulting in maintaining the mucosal mass and integrity and acting on cell production, migration and loss (Wright, et al. 1993).

Thus, the administration of milk peptides might improve the IP.

The evaluation of intestinal permeability (IP) can be useful in the diagnosis and management of many intestinal diseases and systemic pathological conditions. A recognised and widely used method to evaluate the IP in humans is the lactulose/mannitol test, conducted by measuring the percentage of excretion of the two polyols in urines after 5 hours from consumption of a solution containing lactulose and mannitol in 5:1 ratio (Dastych, et al. 2008). The two probe polyols are passively absorbed through the intestine, not significantly metabolised, and excreted in urine in proportion to the quantities absorbed. In particular, mannitol is a polyol which permeates the intestinal mucosa mainly via the transcellular pathway, while lactulose is an oligosaccharide which uses the paracellular way (TJs) (Dastych, et al. 2008); thus, its absorption depends on the integrity of intestinal membrane. Hence, LMR, the ratio of the excretion percentage L% to M% in urine, is considered as a sensitive and accurate indicator of IP. A physiological state is defined by an LMR equal or lower than 0.03, while it is higher for individuals with leaky gut syndrome.

3.1 Bioactive peptides

Bioactive peptides are specific protein fragments consist in 2-20 amino acids, that generally exert a biological activity on the organism (Moller, et al. 2008). They are inactive within the precursor protein in which they are encrypted, and can be release due to food processing or ripening by microbial enzymes (*Lactobacillus* spp) *in vitro,* or gastrointestinal digestion *in vivo* (Gobbetti, et al. 2002; Korhonen and Pihlanto 2007). Their release allows the interaction with body receptors or enzymes, exerting different functions, such as antimicrobial, antioxidative, antithrombotic, antihypertensive, immunomodulatory, mineral binding and opioid (Fig.2) (Shahidi and Zhong 2008).

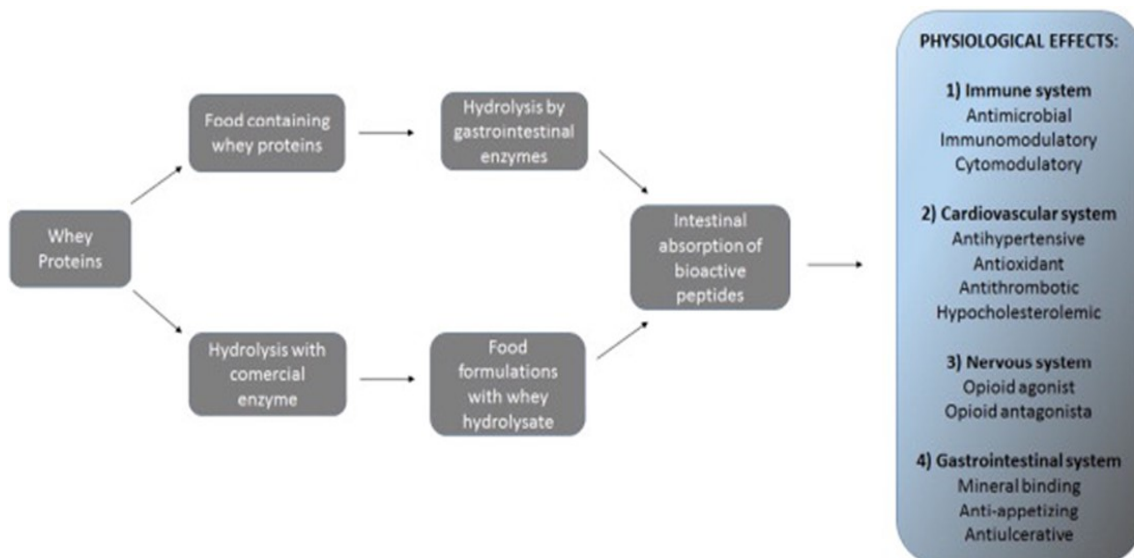

Fig.2 *Mechanism of release and physiological effects of milk peptides*

3.1.1 Sources

The most abundant food source of bioactive peptides are milk (Mohanty, et al. 2016), cheese and dairy products, which we will discuss in detail later. Nonetheless, they can derive from animal (meat, eggs or fish) or vegetal sources (rice, soy, wheat, maize). Among the latter, soy derivatives have been studied and digested by different endoproteases generating ACE inhibitory and anti-thrombotic peptides, mostly belongs to glycinin. Wheat and oat belong to cereal family and their bioactive peptide showed different activities: ACE inhibitory, dipeptidyl peptidase inhibitor, anti-thrombotic, opioid and antioxidative. Another important source is food industry, which produce a large amount of food waste and, nowadays, there is a great interest in finding a functional use of waste in order to avoid the discard. Indeed, a recent study on waste proteins from olive seed treated with different proteases (Esteve, et al. 2015), has generated hydrolysates with antioxidant and antihypertensive activities. Likewise, fruit stones are considered an important waste material of food industry. Gonzalez-Garcia, et al. (2014) studied plum stones and a way to extract proteins from them. Then, the enzymatic digestion produced different hydrolysates with ACE inhibitory and oxidative activities.

3.1.2 Milk: the most important source

Bovine milk, cheese and dairy products are the greatest source of bioactive proteins and peptides among the different above-mentioned sources. Milk contains approximately 3.5% of protein of which 80% are caseins and 20% whey proteins.

Caseins, defined as insoluble fraction, provide the greatest supply of amino acids. Caseins differ from each other in concentration, phosphorus content, amino acid composition, molecular weight and isoelectric point. They are widely known for their mineral binding activity, especially for calcium and phosphorus, increasing their absorption at the gastric level. Unlike whey proteins, rich in branched chain amino acids (BCAA) such as valine, leucine and isoleucine, caseins contain high quantities of histidine, methionine and phenylalanine. Caseins can be divided into 5 fractions:

- αS1-casein: >30% of the total caseins, they have no homologues in breast milk and are the main cause of allergy;
- αS2-casein: >9% of the total caseins, they are present in four isomers which differs in the content of phosphoryl groups;
- β-casein: >28%, is a phosphoprotein with a homologue in breast milk.
- κ-casein: >10%, is the only phosphorylated and glycosylated casein in mammalian milk. This protein exhibits anticoagulant properties, prevents platelet aggregation and promotes the release of serotonin; hydrolysis of k-casein releases glycomacropeptide which reduces the secretion of gastrin and hydrochloric acid.
- γ-casein: > 2% of the total.

Whey proteins, instead, are in a lower concentration and they are also defined as a soluble fraction. They have always been considered as a by-product of the dairy industry, of cheese production, but recently they have been taken into consideration for their biologically activities. Indeed, the application of processes like ultrafiltration and reverse osmosis allowed the development of different concentrated products on the market, such as energy bars, soluble or pre-diluted powder supplements. In addition to the supply of amino acids, these proteins seem to be involved in the protection against infections, in the increase of the immune response and development of the intestine (Kanwar, et al. 2009). They are also an important source of bioactive peptides (Pihlanto-Leppala, et al. 2000).

Whey proteins can be divided into:

- β-lactoglobulin: 51% of whey proteins, is a multifunctional protein consisting of 162 amino acids with a high number of sulphuric residues, it has copper and iron binding properties and inhibits the self-oxidation of fats;
- α-lactalbumin: 22% of whey proteins, it is composed by 123 amino acids including cysteine, lysine and tryptophan and it binds metals such as zinc, magnesium, and cobalt;

- lactoferrin: synthesized by breast epithelial cells, it has the first function of binding iron and transporting it to the intestine;
- lactoperoxidase and lysozyme: they are proteins with antimicrobial activity, the lysozyme is particularly effective against Gram + bacteria, causing lysis;
- Immunoglobulins: they are natural antibodies present in milk with the function of develop the immunity of the new-born baby and they are easily inactivated by heat treatment.

The rest is made up of other functional proteins, such as enzymes and growth factors.

3.1.3 Milk bioactive peptides and their role in human health

Bioactive peptides derived from milk are encrypted into the structure of the parent protein and must be released in order to exert their biological effect. Their release happens in three ways: I) enzymatic hydrolysis during gastrointestinal digestion *in vivo,* where dietary proteins undergo denaturation in the stomach thanks to the action of hydrochloric acid and then they are metabolised into small peptides or amino acids in the small intestine, by trypsin, chymotrypsin and pepsin (Korhonen and Pihlanto 2003); II) *in vitro* microbial fermentation, where proteolytic microorganism such as lactic acid bacteria release bioactive peptides due to the action of intracellular peptidases including endo-peptidases, amino-peptidases, di-peptidases, and tri-peptidases (Christensen, et al. 1999); III) *in vitro* enzymatic hydrolysis of the whole protein by combination of proteinases and bacterial or fungal enzymes (Korhonen and Pihlanto 2003).

3.1.3.1 Antimicrobial peptides

Casein derived peptides have been reported to inhibit the action of pathogenic microorganisms, both gram positive and Gram negative. Chymosin digested bovine caseins release casecidin, a peptide that exhibit antimicrobial activity against Staphylococcus spp., Bacillus subtilis, Diplococcus pneumoniae, and Streptococcus pyogenes (Lahov and Regelson 1996). A specific cleavage of casein with chymosin develop glyco-macropeptide (GMP) and caseinomacropeptide (CMP). The latter may have an inhibitory activity against S. mutans and E. coli whereas GMP modulates the gut microflora (Manso and Lopez-Fandino 2004).

A common structural characteristic to antimicrobial peptides is the presence of a cationic head, necessary for interaction with the negative charges of membrane phospholipids. The whey protein lactoferrin is an iron-binding glycoprotein with antibacterial activity. The hydrolytic action of pepsin on the N-terminal fragment of lactoferrin, originates

lactoferricin, a peptide with strong bactericidal properties. It is characterised by the presence of an intramolecular disulphide bridge and an high proportion of basic amino acids (Kang, et al. 1996), which gives a cationic predominance. The latter allows the formation of ion channels through membrane bilayer (Agawa, et al. 1991), increasing cellular permeability and, hence, killing sensitive microorganisms (Bellamy, et al. 1993).
Lactoferrin and its derivatives show the antibacterial activity *in vitro* against various pathogens, e.g. P. aeruginosa, Clostridium perfringens, C. albicans, Helicobacter pylori, Haemophilus influenzae, Salmonella enteritidis and S. aureus (Farnaud and Evans 2003).
The bactericidal mode of action and the peptide nature of these molecules is very similar to that of the best known bacterial bacteriocins. Some researchers are evaluating the possibility of increasing food safety by adding antimicrobial peptides to foods.

3.1.3.2 Opioid peptides

Opioid-like peptides, once absorbed, prolong gastrointestinal transit time, exert antidiarrheal action, modulate food and electrolyte absorption and insulin secretion. This activity is made possible through the interaction between the bioactive peptides and specific opioid receptors (μ-, δ-, and κ- type) which could be activate by endogenous (enkephalins, endorphins and dynorphins) or exogenous ligands. The formers are normally synthesized by the body and can interact with the opioid receptors thanks to their particular structure, whereas the latter are introduced into the body with the diet. Both types have a common structural feature, namely N-terminal Tyr-Gly-Gly-Phe or -Tyr. sequence. The phenolic hydroxyl group of tyrosine has a negative potential essential for opioid activity, indeed its lack results in a total absence of bioactivity (Chang, et al. 1981).
Milk proteins represent an important source of opioid peptides (Teschemacher 2003). Specifically, most of the identified peptides are μ receptor agonists, while others may act as antagonists. The first food-derived opioid peptide identified were the β-casomorphins (Brantl, et al. 1979), as a fragments of bovine β-casein sequence 60-70 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu): β-casomorphins-5 and -7 (Svedberg, et al. 1985). These molecules have been characterized mainly as μ type opioid receptor ligands and their presence in humans has been demonstrated in the intestine after intake of cow's milk (Svedberg, et al. 1985). Bovine and human α-lactalbumin and β-lactalbumin generate two opioid agonist peptides, α-lactorphin (Tyr-Gly-Leu-Phe $\bullet NH_2$) and β-lactorphin (Tyr-Leu-Leu-Phe $\bullet NH_2$) respectively (Chiba and Yoshikawa 1985).

Opioid antagonist-acting peptides are casoxin C and lactoferroxins, derived from bovine and human κ-casein and human lactoferrin, respectively. The former has, *in vitro*, a prokinetic effect on the muscle cells of the guinea pig ileum, while the latter has been shown to have activity very similar to naloxone when obtained by digestion with pepsin and extraction with chloroform and ethanol.

Furthermore, gastrointestinal digestion of caseins must develop the aforementioned peptide sequences in order to exert their biological activities. Considering that their absorption through the intestinal epithelium has not been proved, opioid peptides act especially in situ, modulating the intestinal transit, amino acids uptake and the water balance. Even if a minimal amount of these peptides were absorbed, it would be rapidly degraded by esterases and peptidases present in the circulatory system (Meisel 1997).

New-borns, instead, have a greater intestinal permeability that could allow a higher absorption of β-casomorphins, with a consequent systemic effect (Sturner and Chang 1991).

3.1.3.3 Immunomodulatory peptides

The immune system is a complex system of specialised cells with the sole function of protecting the body from harmful substances and events. After years of studies, today we can assert that diet plays a very important role as regards the modulation of the immune system. In particular, some peptides are able to stimulate the immune response. The molecular mechanisms underlying this effect are unclear, but some peptides have been shown to stimulate both cell-mediated and humoral activity.

During gastrointestinal digestion, bovine and human caseins release different peptides with immunostimulatory activity. Jolles, et al. (1981) were the first to prove that breast milk subjected to tryptic hydrolysis has a strong immunostimulatory activity.

Among casein-derived peptides, the fragment f54-59 of human β-casein isolated from milk tryptic hydrolysate (Parker, et al. 1984) generate the Val-Glu-Pro-Ile-Pro-Tyr peptide, very effective in stimulating *in vitro* the phagocytosis of both human and murine macrophages and also increasing resistance to Klebsiella pneumoniae infection in mice (Migliore-Samour, et al. 1989). The fragments f63-68 and f191-193 of bovine β-casein, and f194-199 of αS1-bovine casein may affect phagocytizes in humans *in vitro* (Migliore-Samour and Jolles 1988). Moreover, lactoferricin B, obtained from lactoferrin digested, showed a direct bind to neutrophils.

The mechanism by which milk derived peptides exert their immunomodulatory effects is not yet defined. Nonetheless, we can hypothesise a relationship between opioid and

immunomodulatory peptides because of the presence of opioid μ receptors for endorphins on human leukocytes and T lymphocytes (Lopker, et al. 1980; Wybran, et al. 1979).
The binding site recognized by membrane receptors could be an arginine residue at the N- or C-terminal region (Paegelow and Werner 1986).

3.1.3.4 Antithrombotic peptides

Clare and Swaisgood (2000) demonstrated that there are similarities between the blood clotting process (interaction of fibrinogen and thrombin) and the milk clotting (interaction of k-casein with chymosin). Casoplatelin, a undecapeptide f106-116 derived from bovine κ-casein, have three amino acid residues (Ile-108, Lys-112, Asp-115) in homologous position as compared with the C-terminal side of γ chain of fibrinogen (Jolles, et al. 1978). This can inhibit the aggregation of ADP activated platelets and compete with the binding of human fibrinogen γ chain to a specific receptor on the platelet surface.

Moreover, the f103-111 fragment of bovine κ-casein is able to prevent blood clotting by inhibiting platelet aggregation, although it is not able to interfere with the binding of fibrinogen with ADP activated platelets (Fiat, et al. 1993). Therefore, by hindering the formation of the clot, these peptides may be of interest to prevent heart and brain disorders.

3.1.3.5 Antioxidant peptides

The oxidation of food constituents is a key event in the deterioration of food. Lipid peroxidation generate free radicals and secondary lipid peroxidation products that can lead to the breakdown of fatty acids and the consequence cell damage. Free radicals can also modify DNA, proteins and small cell molecules and are believed to play a significant role in the onset of diseases, such as cardiovascular disease, diabetes mellitus, neurological disorders and Alzheimer's disease (Stadtman 2006). Hence, food oxidation is responsible of reduction of food nutritional value and safety, producing unwanted flavours and toxic substances. Therefore, it is important to delay lipid oxidation and free radicals generation in foods containing lipids and/or fatty acids (Peng, et al. 2010).

Considering that synthetic antioxidants such as butylhydroxytoluene, butylhydroxyanisole and propyl gallate has been limited due to their potential toxic effects on humans, the use of natural bioactive peptides as antioxidants is in great demand (Zhang, et al. 2009).

Protein hydrolysates peptides act as antioxidants through different mechanisms: inactivation of reactive oxygen species (ROS), free radicals' degradation, lipid peroxidation inhibition, metal ions chelation, or a combination of these mechanisms. The responsible of the antioxidant mechanism activity is the peptide (Phelan, et al. 2009).

Therefore, considering a protein hydrolysate as a complex mixture of peptides and amino acids, distinct antioxidant mechanisms can act concurrently.

The antioxidant activity of the peptides generated by proteolytic hydrolysis of proteins is related to a greater number of ionizable groups and also to the exposure of hydrophobic groups, such as proline, histidine, tyrosine or tryptophan (Sarmadi and Ismail 2010).

Wang and De Mejia (2005) have deepened the antioxidant characteristics of soy amino acids and have indicated tyrosine, methionine, histidine and lysine as amino acid residues with antioxidant activity. Tyrosine has a ring aromatic structure which can donate a proton to an electron deficient species like radicals. Non-polar amino acids, such as methionine, can improve the solubility of peptides in a lipid matrix enhancing the accessibility of hydrophobic radical species or polyunsaturated fatty acids.

Most of the identified milk antioxidant peptides derived from α-casein and may influence free radical scavenging activity and inhibit enzymatic and non-enzymatic lipid peroxidation (Rival, et al. 2001). In particular, antioxidant peptide with methionine or tyrosine fragments has a higher radical scavenger activity (Torkova, et al. 2015).

3.1.3.6 ACE-inhibitory peptides

Different bioactive peptides derived from food proteins have been isolated and evaluated for their antihypertensive activity in order to avoid the unwanted side effects of synthetic antihypertensive drugs. Indeed, despite the synthetic ACE inhibitors (captopril, enalapril and lisinopril) are remarkably effective in regulating blood pressure and commonly used in clinic (Ondetti, et al. 1977), they show different side effects, such as allergic reactions, rashes, coughs and taste disorders (Bougatef, et al. 2009). Therefore, research for safer, cheaper, and more innovative non-toxic ACE inhibitors has proven to be necessary for the control and treatment of hypertension.

Thus, food scientists and technologists focused on the bioactivity of casein and whey proteins peptides, since milk proteins contain in their primary structure amino acid sequences which, if released by hydrolysis or fermentation processes, exert significant antihypertensive activity (Erdmann, et al. 2008).

ACE is a zinc-metal protease found in different tissue throughout the body, responsible for converting Angiotensin I to a potent vasoconstrictor angiotensin II, so raising the blood pressure. Inhibition of ACE reduces the angiotensin II production and the degradation of bradykinin, a vasodilator peptide, with the results of lowering the blood pressure.

Casein represent a very important source of peptides with ACE inhibitory activity. In particular casokinins represent different fragments of human and bovine caseins obtained

by tryptic hydrolysis, such as bovine αS1-casein (fragments 23-24, 23-27 and 194-199) and β-casein (fragments 177-183 and 193-202) (Maruyama, et al. 1987).

The β-casein-derived lactotripeptides Val-Pro-Pro and Ile-Pro-Pro, f (84–86) and f (74–76), were the most powerful ACE inhibitory peptides identified in fermented milk with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura, et al. 1995).

Whey proteins α-lactalbumin and lactoglobulin, instead, generated the opioid sequences α-lactorphin and β-lactorphin peptides that have also been identifies as ACE-inhibitory peptides (Maruyama, et al. 1987; Maruyama, et al. 1985).

C-terminal tripeptide sequence is responsible to the binding to ACE, which prefers hydrophobic amino acids residues such as proline, lysine, or arginine.

3.1.3.7 Mineral-binding peptides

About 30% of the phosphorous of milk is bound via monoester linkages to seryl residues of casein, forming caseinophosphopeptides (CPPs). The phosphoric acid is important both for the structure of the protein and for its function because it is able to bind calcium and magnesium ions, exert a carrier function.

CPPs, released during gastrointestinal digestion, act as biocarriers and play an important role in regulating mineral balance in the body, especially about the calcification process of bones. The sequestering action of the CPPs, with the formation of soluble complexes easily absorbed, avoid the precipitation and therefore the loss of calcium in the form of phosphate (Sato, et al. 1986). CPP can also have an anti-carcinogenic effect by promoting both recalcification of tooth enamel and by inhibiting the adhesion of plaque forming bacteria. The addition of CPP's to tooth paste formulas has been suggested to have anti-cariogenic effects and to prevent enamel demineralization (Nagpal, et al. 2011).

Contrary to what might have been expected, it has been shown that CPPs are not only present in the stomach and duodenum (acidic pH), but also in the small intestine of man (pH values from slightly acidic to alkaline). This discover underlying that the alkaline pH conditions of the small intestine help the activity of phosphatase, which promotes the activation of enzymatic cascades and an increase in calcium absorption.

A large number of CPPs deriving from *in vitro* hydrolysis or *in vivo* digestion of αS1, αS2, and β vaccine caseins are currently known (Kitts 1994): f43-58, f45-55, f59-79, f66-74, and f106-119 from αS1; f2-21, f46-70, f55-75, f126-136, and f138-149 from αS2; f1-25, f1-28, and f2-28 from β casein.

CPPs are characterised by three phosphate groups as monoesters of serine residues, followed by two glutamic acid residues, SerP-SerP-SerP-Glu-Glu (Meisel 1997). This

common structural element represents the binding site for minerals (Meisel 1998) and the consequent cause of high resistance to proteolytic phenomena cause of degradation (Kasai, et al. 1995).

Lastly, CPPs bind many elements, in addition to calcium, from the most present magnesium and iron, to trace elements, such as zinc, barium, chrome, nickel, cobalt and selenium. Otherwise, the structural properties necessary for this activity is not yet fully understood. In most cases, CPPs are low molecular weight peptides (up to 9 residues) rich in hydrophobic amino acids and characterised by the presence of proline, lysine, or arginine on the *C*-terminal end.

3.1.3.8 Antidiabetic peptide

Oral administration of whey proteins and their hydrolysates has been reported to positively influence blood sugar control with insulinotropic responses in humans (Jakubowicz and Froy 2013). Therefore, their activity is recognised for T2DM, i.e. a metabolic disorder characterised by a reduced secretion of insulin by pancreatic β cells and insulin resistance in tissues.

Although not fully understood, the effects on blood glucose appear to be mediated, *in vivo*, by whey proteins bioactive peptides and branched-chain amino acids that would induce the synthesis of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Nilsson, et al. 2007), and the inhibition of dipeptidyl peptidase IV (DPP IV) (Drucker 2006).

3.1.3.8.1 DPP IV inhibition

Food ingestion stimulate GLP-1 and GIP release in the gastrointestinal tract, two hormones called incretins which contributes up to 70% of insulin release in healthy subjects. In T2DM the action of GLP-1 appears to be preserved, although the incretin effect is severely depressed. GLP-1 and GIP are substrates of DPP IV, a ubiquitous enzyme able to degrade them quickly, resulting in a rapidly decrease of their plasma levels. Thus, DPP IV inhibitors could increase the half-life of active GLP-1, enhancing the insulinotropic effect and glycaemic control (Power, et al. 2014).

Different food proteins are considered precursors of peptides inhibitor of DPP IV, such as whey, cow, sheep, and goat milk proteins.

3.2 Buffalo mozzarella cheese

Buffalo mozzarella cheese is a fresh table cheese of soft spun dough, derived from whole buffalo milk. It is the most famous and consumed product of central-southern Italy.

Particularly, the so called “Mozzarella di Bufala Campana” is listed as a protected origin designation (POD) product from the European Council [Commission Regulation (EC) No. 1107/1996)]. Its disciplinary of production names 96 municipalities in Southern Italy as the only places of production of the so called “Mozzarella di Bufala Campana DOP”. It shows a peculiar manufacturing process that contemplates exclusively use of fresh whole buffalo milk characterised by a specific macronutrient composition, and precise chemical-physical and organoleptic properties of the final product.

Therefore, only the mozzarella produced following the aforementioned specification can be portrayed with the symbol of the relative "Consortium for the Protection of Buffalo Mozzarella from Campania" (Fig.3), born in 1981 for the protection, enhancement, promotion and supervision of this singular cheese.

Fig. 3 *Consortium for the Protection of Mozzarella di Bufala Campana PDO logo*

The consortium purpose is to protect the production and trade of Mozzarella di Bufala Campana, to defend the denomination itself, both in Italy and abroad, in compliance with the Controlled Designation of Origin (DOC) production regulations (DPCM 10/05/1993) and the PDO. It exerts a constant supervision of supply chain quality. The trademark of the *Protection Consortium* affixed to the wrapping, with a sign of the legal details and the authorization number released by the same, is a guarantee of authenticity of typicality of the product and the geographical origin.

3.2.1 The PDO brand

Mozzarella di Bufala Campana obtained in 1996 the "Protected Designation of Origin", that is the prestigious European brand with which the organoleptic and product characteristics are recognised. The latter are mainly derived from the environmental conditions and traditional processing methods existing in the specific area defined by the PDO specification.

Consequently, the European Commission has created an ad hoc logo (Fig.4) that allows the identification of food products included in the protection systems, in order to recognise manufacturers products to consumers.

Fig.4 *Protected Designation of Origin logo*

To be able to receive the PDO designation, the must have production conditions for raw materials and their transformation must exist until the product is finished. These processes must therefore be carried out in the region delimited by the specification.

In other words, a PDO cheese must be made with milk from farms in the area limited by the PDO and must have the qualities and characteristics exclusive of the geographical environment of origin. By "geographical environment", the law means the climate and soil quality, but also technical knowledge and local artisans. Therefore, with the PDO introduction, milk, raw material, and final product characteristics are established, but above all, the PDO geographical areas of production.

Thus, the area of origin of the milk for processing "Mozzarella di Bufala Campana" cheese, includes 96 municipalities among Campania, Lazio, Puglia, and Molise regions.

3.2.2 Buffalo milk

Buffalo milk is richer in protein, fat, and calcium content than other animal species, such as cow or sheep. Another singular characteristic of buffalo milk is the absence of carotenoids in its composition, characterised by the typical white porcelain colour of finished product.

Buffalo milk has an opaque white colour, the fat content is between 6-9% with prevalence of oleic acid among unsaturated acids, and of palmitic acid among saturated acids. The nitrogenous substances correspond to 3.8-4% (albumin, globulin).

The main chemical-physical differences between buffalo and vaccine milk are represented by fat and protein content. They are on average 7.5% and 4.4% respectively in buffalo milk and 3.3% and 2.7% in cow's milk.

Another typical aspect of buffalo milk is its microbiological nature. Indeed, under normal conditions, there are some strains of *lactobacillus spp.* in higher concentrations than those contained in cow's milk. The metabolic activity of these bacteria would be responsible, for the most part, for the typical flavour and aroma of this cheese.

These different values contribute to the typicality of the product, thanks to the different finished product yield. Indeed, mozzarella cheese from buffalo milk has a yield an average of 24.6%, while cow's milk gives a yield of 13%.

Although the production of buffalo milk continues throughout the year, its monthly distribution varies; there is a greater availability in autumn and winter months and a strong contraction in summer months, because of the reproductive characteristics of the buffalo. Nowadays, there are advantageous techniques for seasonally adjusting and guaranteeing the delivery in spring of at least 20-30% of the buffaloes.

3.2.3 Mozzarella di Bufala Campana PDO production

3.2.3.1 The filtering of milk

Buffalo milk must be delivered to farms within 12 hours of milking, then stored in suitable containers, stainless steel, which do not change the organoleptic characteristics. Before being processed, the milk is filtered in order to remove all the impurities.

3.2.3.2 Milk coagulation

Natural whey-graft, obtained by leaving the whey of the previous day to spontaneously acidify, is added to buffalo milk at room temperature. Coagulation is carried out by adding liquid calf rennet to buffalo milk, in proportions generally 10.000:1. The heating of milk occur by direct introduction of steam, then rennet is added (18-20 ml/quintal of milk). The optimal temperature range is between 34 °C and 38 °C and the average duration of coagulation does not exceed 30 minutes.

3.2.3.3 Curd: break and ripen

The breaking of the curd is usually carried out manually with a wooden stick at the end of which a wooden disc with the convex external face is fixed, and pushed until cheese-like lumps are obtained, with dimensions of 3-6 cm. The braking occurs in two phases: the first reduces the curd into cubes and, after a stop of about 30 minutes, there is the second break with the wooden stick. Then the curd is cut into large slices with a knife or traditional sickle and placed on a

draining table to ripen for further 15 / 30 minutes. Consequently, curd breaking derivatives are left to acidify under whey for on average of 3 / 4 hours.

The latter is one of the process variables that most influence the quality of the Mozzarella. The right degree of ripening of the curd is determined by the "empirical spinning test". The procedure is as follows: about 100g of matured paste are melted in hot water, the melted pasta is placed on a stick and pulled with hands. Then you can continue to the spinning only if the dough stretches in continuous filaments without breaking, and longer than one meter.

3.2.3.4 Spinning

This processing stage most affects the consistency and quality of the finished product and the processing yield. In traditional processing, the spinning of the dough is still done manually. The mature pasta is cut into thin slices with a chopped-curd and placed in a wooden vat, in which it is melted by adding boiling water. Subsequently, with the help of a bowl and a wooden stick, the melted dough is and pulled, until a homogeneous and shiny dough is obtained.

3.2.3.5 Mozzarella moulding

The moulding of buffalo mozzarella cheese is carried out manually by two operators, of which one with the thumb and index finger detaches ("mozzatura") pieces of spun paste from a globular mass of about 2 / 3 Kg, and the other operator supports it; hence the name "mozzarella". The spun paste is handled with great care and experience, carrying out characteristic movements that end with the docking.

3.2.3.6 Salting

Salting is carried out by immersing Mozzarella in successive saline solutions with a different concentration of salt, from 10 to 18%. Once the mozzarella is extracted from the brine and immersed in the spinning water, the so called “government liquid”, the concentration of salt in the cheese tends to balance. Indeed, because of a diffusion process, the concentration of salt is lowered in the outer layers of the cheese, while rising in the inner ones.

3.2.4 Preservation

Mozzarella di Bufala Campana PDO is composed only of natural products (milk, salt, rennet). There are no preservatives and it is only immersed in "government liquid", to which salt and diluted whey are added. This type of liquid is essential because it gives the right salting tone, enhancing its flavour and organoleptic qualities. It is suitable for extending the shelf-life, ensuring its correct conservation.

Mozzarella is always stored immersed in its liquid at room temperature, until ready to consume it.

3.2.5 Nutritional values

Mozzarella di Bufala Campana is an easily digestible cheese, with a reduced lactose and cholesterol content, an excellent source of protein with a high biological value and a moderate fats intake (Table 4). In addition, the cheese supplies high quantities of calcium, phosphorus, water-soluble vitamins, such as B1, B2, B6 and niacin. It is also a good source of vitamin E and zinc, substances that help to contrast the negative action of free radicals.

	Energy value	Proteins	Carbohydrates	Fats	Phosphorus	Calcium
Amount per serving (100g)	246,4 Kcal	16,2 g	0,4 g	20 g	320 mg	245 mg

Table 4 *Nutritional values of Mozzarella di Bufala Campana*

4 Diabetes

Diabetes is a chronic disease characterised by the presence of high blood glucose levels, hyperglycaemia, compared to physiological values (normal reference values of glucose measured by fasting examination are between 60 and 110 mg/dl) due to an altered insulin production or function. Insulin is a hormone produced by the pancreas, which allows glucose to enter cells and its consequent use as energy source. When this mechanism is altered, glucose collect in the bloodstream (Assoc 2013).

To date, diabetes is classified into type 1 (T1DM) and type 2 diabetes mellitus (T2DM). According to the Ministry of Health, in Italy there are about 300.000 patients with T1DM, a number that is expected to increase. It affects about 10% of people with diabetes and generally occurs in childhood or adolescence. In T1DM, pancreas does not produce insulin due to the destruction of the pancreatic β cells that, physiologically, produce this hormone. Therefore, the therapy consist of subcutaneous insulin injection, every day and for life (Assoc 2013). According to data released by the Italian Ministry of Health, patients with T2DM are 5.5% of the population, or more than 3 million, to which it is added one million people to whom the disease has never been diagnosed. T2DM is the most common form of diabetes and represents 90% of cases.

The aetiology is still unknown, nonetheless patients with T2DM normally produce insulin by pancreatic β cells, but the body's cells are unable to use it. Typically, the disease occurs after the age of 30-40 and numerous risk factors have been recognised associated with its onset. Among them, there are familiarity, poor exercise, overweight and belonging to some ethnic groups. Regarding familiarity, about 40% of type 2 diabetics patients have first degree relatives (parents or siblings) affected by the same disease, suggesting a strong hereditary component for this type of diabetes (Assoc 2013).

The pharmacological treatment of T2DM involves a therapy based on oral hypoglycaemic drugs, a large class of molecules able to reduce blood sugar through different mechanisms, such as increasing insulin secretion, reducing insulin-resistance, slowing down intestinal glucose absorption, and thus increasing its renal elimination. Oral anti-diabetics drugs currently available on the market are as follows: biguanides, sulphonylureas, glinides, thiazolidinediones, DPP-4 enzyme inhibitors, intestinal α-glucosidase inhibitors and renal sodium-glucose transport protein 2 (SGLT-2) inhibitors (Nathanson and Nystrom 2009).

The side effects associated with antidiabetic therapies depending on the drug. In general, therapy with oral medications can trigger gastrointestinal, skin, blood and visual problems.

In some cases, an increase in liver enzymes may also occur. Oral antidiabetics can also interfere with other medications. Sulphonylureas, for example, reduce the effectiveness of diuretics, oestrogens and rifampicin, while increasing that of sulphonamides. Metformin, a widespread biguanide, enhances the effect of anticoagulants, while reduces vitamin B12 absorption (Cheng and Fantus 2005).

Considering the ABA function in glycaemic homeostasis, as reported above, the physiologically mediated response of this hormone could be compromised in diabetes mellitus.

4.1 Abscisic acid

4.1.1 Vegetal world

Abscisic acid (ABA) is an isoprenoid compound naturally present in fruits and vegetables identified for the first time in plants in the late 1960s, in relation to the wilting and stomata closure (Nambara and Marion-Poll 2005).

Nowadays, ABA is recognised as a phytohormone involved in the adaptive response to a wide variety of environmental stresses, such as drought, high temperatures, refrigeration and salinity (Qin and Zeevaart 2002). ABA is also known as a suppressive of plant growth regulators, by inducing expression of cell cycle inhibitors, effective on DNA and protein synthesis, and, thereby, arresting cell divisions (Wang, et al. 1998) and blocking cell cycle progression at the initial stages (Swiatek, et al. 2002).

Furthermore, ABA plays a role in the induction and maintenance of seed dormancy, where the highest ABA concentrations are recorded (Seo and Koshiba 2002), and in fruits ripening (Leng, et al. 2014). Generally, typical changes of ripening are identified with colour change, through the accumulation of chlorophyll, carotenoids and/or flavonoids; structural modification, such as alteration of cell turgor, cell wall structure and /or metabolism; change in sugars, acids and volatile compounds that affect the nutritional quality, flavour and aroma and, finally, there is a greater susceptibility to microorganisms, probably related to the loss of the integrity of the cell wall (Giovannoni 2004). At the beginning of fruit ripening, there is a strong accumulation of ABA until a specific stage after full flowering, to then reach the minimum levels in the full ripening phase, immediately preceding the fruit harvest. In addition, Lacampagne, et al. (2010) demonstrated that ABA leads to anthocyanin synthesis, necessary for colouring and defending the fruit against potential damage, thanks to the radiation filter and antioxidant actions.

In summary, this effect would be highly requested by the fruit at an immature stage when cell cycle progression can be disturbed by several environmental factors, such as oxidative stress (Swiatek, et al. 2002). Later, the production of increasing levels of protective compounds, such as antioxidants, would make possible the completion of fruit development, so that the action of ABA is no longer required (Swiatek, et al. 2002).

4.1.2 Animal world

Since its discovery, ABA has been identified in numerous plant and non-plant species, including mammals. Indeed, a study of 1980s carried out on pigs and rats identified the presence of the phytohormone within the tissues of these animals, particularly high in the brain (Lepagedegivry, et al. 1986). Specifically, in the brain of rats fed with low ABA diet there were higher concentrations of the hormone than those found in the control group. This result allowed to hypothesise the endogenous synthesis of ABA in mammals.

To date, numerous studies carried out on mouse and human cell models have confirmed that following different kind of stimuli, it is observed the synthesis and release of ABA in granulocytes (Bruzzone, et al. 2006), monocytes (Magnone, et al. 2009) and macrophages (Magnone, et al. 2012), pancreatic β-cells (Bruzzone, et al. 2008), mesenchymal stem cells (Scarfì, et al. 2008), progenitor hematopoietic pathways (Scarfì, et al. 2008) and adipocytes (Bruzzone, et al. 2012).

ABA effect at the cellular level highlight its role when environmental conditions change and lead to the hypothesis of the conservation of ABA role in the adaptive response to stress at early stage of evolution, both in plants and animals (Zocchi, et al. 2001). This hypothesis arises from the similarity of the ABA signal transduction pathway in plants and animals, which provides an increase in the concentration of intracellular calcium stimulated by cyclic adenosine diphosphate (ADP) ribose. For example, the mammalian cells most exposed to environmental stimuli are granulocytes. They are a first defence against pathogens, with phagocytic activity, oxygen species (ROS) and nitric oxide (NO) production dependent on the increase in intracellular calcium concentration mediated by cyclic ADP-ribose. The signalling pathway triggered by ABA in granulocytes involves the lanthionine synthetase C-like protein 2 (LANCL-2), a receptor coupled to the Gi protein (Sturla, et al. 2009), identified and characterised as ABA receptor in mammals and humans. This interaction starts a transduction signalling cascade, which activates adenylate cyclase with the consequent cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA)-mediated phosphorylation of ADP-ribosyl cyclase. The latter determines the increase in cyclic ADP-ribose which mediates the increase in the

intracellular calcium. In addition, ABA also triggers phospholipase C pathway which increase inositol triphosphate production and, therefore, the cytoplasmic calcium.

Thus, ABA influence the function of human granulocytes, modifying the intracellular concentration of calcium, stimulating phagocytosis, ROS and NO production, chemotaxis and has therefore been recognised as an endogenous pro-inflammatory cytokine in granulocytes (Bruzzone, et al. 2006).

Considering that stressful conditions such as trauma, infections or fever induce a state of hyperglycaemia, and considering that the second messengers of ABA signalling pathway in granulocytes, cAMP and cyclic ADP-ribose, are also recognised as mediators of glucose-induced insulin secretion (Charles, et al. 1973; Takasawa, et al. 1993), ABA may play has been proposed for ABA to play a role in regulating glucose homeostasis.

4.1.3 ABA and regulation of glycaemic homeostasis

The first evidence regarding the involvement of ABA in glucose homeostasis in mammals has been elaborated on the basis of the structural similarity of the hormone with thiazolidinediones, a class of insulin-sensitising antidiabetic drugs.

Guri, et al. (2007) showed, in genetically obese db/db mice (leptin deficiency) fed with high fat diets, a significant improvement in the normalization capacity of glucose after an intraperitoneal glucose tolerance test, due to the ABA agonist action on peroxisome proliferator-activated receptor γ (PPARγ).

Bruzzone, et al. (2008) demonstrated that nanomolar concentrations of ABA potentiates insulin release by murine and human cells, using the same signalling pathway that involves cAMP, PKA and cyclic ADP-ribose. In addition, the ability of the insulin-secreting cells themselves to produce and release ABA has been tested with positive results. In short, pancreatic β-cells release ABA in response to glucose and ABA, in turn, increases and stimulates the secretion of glucose-dependent and glucose-independent insulin, suggesting the existence of an autocrine/paracrine *feed-forward* cycle (Bruzzone, et al. 2008).

In a subsequent study (Bruzzone, et al. 2012), it was found that glucose assumption increase the concentration of plasmatic ABA in healthy human subjects. In all participants, who underwent the oral glucose tolerance test (OGTT), the increase in ABA levels above the basal values was recorded from 15 to 60 minutes after the glucose load. Considering that the administration of intravenous glucose in the same subjects did not show the same results, the authors evaluated a stimulating effect of incretins on ABA release. Incretins are a group of metabolic hormones produced at gastrointestinal level, which secreted after meals by enteroendocrine cells (L cells), stimulate insulin secretion by pancreatic β cells

and inhibit glucagon secretion. Thus, ABA induce the release of GLP-1 through a cAMP / PKA-dependent mechanism and activates the GLP-1 transcription factor. These results suggest a positive feedback mechanism between ABA and GLP-1 activated by hyperglycaemia, where ABA stimulates the production of GLP-1 by L cells (Bruzzone, et al. 2015) and GLP-1 stimulates the endogenous release of ABA from β cells, which in turn further stimulates insulin secretion.
Finally, ABA also induce *in vitro* glucose uptake in human adipocytes and murine myoblasts by increasing the translocation from the intracellular vesicles to the plasma membrane of GLUT4 through protein kinase B phosphorylation (Bruzzone, et al. 2012). The entire mechanism of action of ABA is illustrated in Fig.5.

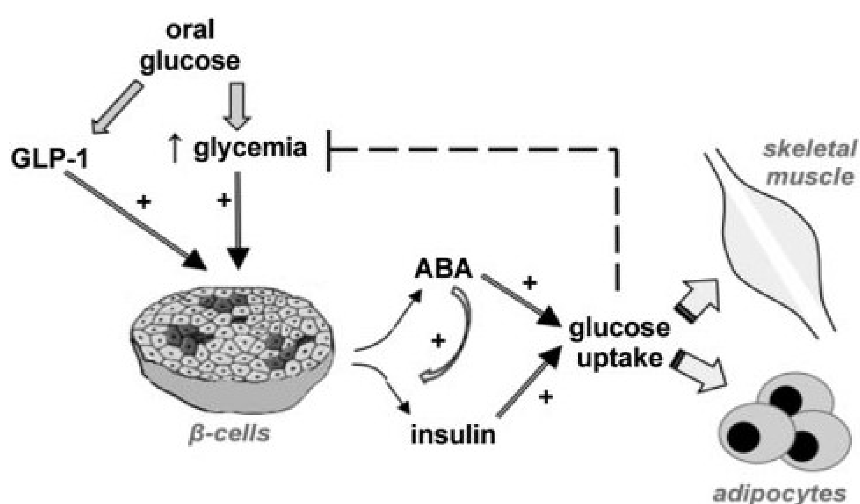

Fig.5 *Schematic representation of ABA role in regulating blood sugar.*

4.2 Peschiole (green peaches) as ABA source

Fruit thinning is a widespread agronomical practice that involves removing excess fruits to produce better-sized, ripe and healthy fruits, albeit in smaller numbers. In fact, when subjected to a very high load, plants consume small and poor-quality fruits, which instead is clearly improved following the thinning process. Among other advantages, this practice provides sunlight and air to penetrate the branches, thus improving the uniformity of ripening, as well as limiting the breaking of the branches themselves and the spread of pests and diseases. Generally, fruit thinning is generally applied to a specific range of tree fruits, including apples, pears, plums, peaches and nectarines, and consists in leaving from a minimum of one fruit every 5-8 cm (plums and apricots) to a maximum of one fruit every

10-15 cm (apples and pears) and 20-25 cm (peaches and nectarines) on tree branches (Royal Horticultural Society, https://www.rhs.org.uk/advice/profile?PID=340).

Since this practice may interest up to 40% of the entire tree fruit load, fruit thinning may lead to a massive agricultural waste product which is generally destined to fertilising or feeding. Occasionally, these waste fruits are recovered by the food industry to produce specific preserves, such as the pickled “peschiole” (green peaches) of Vairano Patenora, a typical Southern Italy food product consumed as appetiser (Fig.6). They are small peach fruits that are picked while still green at the first thinning, when they measure just one or two centimetres and the seed has not hardened yet. They belong to the category of peach-hazel, with a hairless fruit, and are particularly apt for following preparation. After being carefully washed, peschiole are cooked in water and vinegar with spices, and then kept in glass jars. Then, they are preserved in vinegar and are eaten as an aperitif, characterised by a decisive and strongly taste, and a crunchy and firm consistency. Only recently this ancient recipe has been used outside of a family circle and has made peschiole famous, especially in restaurants and in gastronomic establishments specialised in traditional products.

In an immature development stage these waste fruits are supposed to be a significant source of ABA, which could be of potential interest both to the food and nutraceutical industry.

Fig.6 *Peschiole, from thinning practise (20-25 days after full flowering) to food product.*
(Source: Campania Region, Agriculture Department)

5 Plasma triglyceride lowering effect by ChiaCor, a novel chia seed-based nutraceutical formulation: preliminary results from a randomised clinical trial

5.1 Introduction

Although many types of oily seeds are indicated as a good source of ω-3 FA, and strongly suggested to be daily consumed for a proper intake of such precious cardioprotective nutrients, some of them, such as the same flax seeds and chia seeds, are characterised by a very small size, so to be quite difficult, or almost impossible, to be properly chewed and, thus, crushed. This aspect could at the base of a very low bioaccessibility of seed ω-3 FA. What is more, some oily seeds are characterised by a very high capacity of hydration: chia seeds, for example, can absorb up to 27 times their weight in water, forming a gelatinous mass which, on one side, usefully contributes to stimulate the intestinal peristalsis, but, on the other hand, provides a very low, or even null, bioaccessibility of ω-3 FA (Goh, et al. 2016). A preventive grinding before the assumption of such oily seeds could be expected to favour the bioaccessibility of ω-3 FA. Nevertheless, it is extensively reported that the effects of gastrointestinal digestion on vegetable and animal oils, not only lead to a significant degradation of ω-3 FA, thus, much decreasing their actual intestinal bioaccessibility (Cofrades, et al. 2017; Domoto, et al. 2013; Nieva-Echevarría, et al. 2017), but, more importantly, also favour the development of oxidation products which would be absorbed along the duodenal tract (Maestre, et al. 2013). Thus, the aim of the present work will be to develop a novel nutraceutical formulation, based on gastric-resistant (GR) micronized chia seeds and antioxidative co-formulants. Specifically, this formulation will be tested for its potential effects on human plasma TG levels through a randomised clinical trial.

5.2 Materials and methods

5.2.1 Reagents and standards

All chemicals and reagents used were analytical reagent. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use. Chemicals and reagents used to simulate the gastrointestinal digestion were: potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts (Sigma Chemical Co., St. Louis, MO, USA).

5.2.2 *Chia seed-based nutraceutical formulation*

The nutraceutical formulation used in this study consisted of GR capsules containing cryo-micronized chia seeds (500 mg/cps) and vitamin E (15 mg/cps). Cryo-micronized chia seeds were purchased by MB-Med Company (Turin, Italy). The product was formulated by the Department of Pharmacy, University of Naples "Federico II" (Naples, Italy), and registered with the name of ChiaCor.

5.2.3 *In vitro gastrointestinal (GI) digestion*

The assay was performed according to the procedure described by Raiola, et al. (2012), with slight modification. GI digestion was distinguished into salivary, gastric and duodenal digestive steps. The following samples were submitted to GI digestion: sample 1, 500 mg chia seeds; sample 2, 500 mg cryo-micronized chia seeds; sample 3, 1 GR capsule containing cryo-micronized chia seeds (500 mg); sample 4, 1 GR capsule containing cryo-micronized chia seeds (500 mg) and vitamin E (15 mg). For the salivary digestion, the samples were mixed with 6 mL of artificial saliva composed of: KCl (89.6 g/l), KSCN (20 g/l), NaH_2PO_4 (88.8 g/l), Na_2SO_4 (57.0 g/l), NaCl (175.3 g/l), $NaHCO_3$ (84.7 g/l), urea (25.0 g/l) and 290 mg of α-amylase. The pH of the solution was adjusted to 6.8 with HCl 0.1 N. The mixture was introduced in a plastic bag containing 40 ml of water and homogenised in a Stomacher 80 Microbiomaster (Seward, Worthing, UK) for 3 min. Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted to 2.0 with HCl 6 N, and then incubated at 37 °C in a Polymax 1040 orbital shaker (250 rpm) (Heidolph, Schwabach, Germany) for 2 h. After the gastric digestion, the pancreatic digestion was simulated as follows: the pH was increased to 6.5 with $NaHCO_3$ 0.5 N and then 5 ml of a mixture pancreatin (8.0 mg/ml) and bile salts (50.0 mg/ml) (1:1; v/v), dissolved in 20 ml of water, were added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. After each step of digestion, 10 ml of the obtained extract were centrifuged at 4000 rpm and 4 °C for 1 h: before each following step, the digestion procedure was started over again. To determine the peroxide values and the polyunsaturated fatty acids (PUFA) quali-quantitative profile, the intestinal digestive solution has been freeze-dried and, then, subjected to lipid extraction according to AOAC (1995) method 948.16 (WINDHAM 1995), by using a 6-place units Extraction Unit E-816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000 g for 5 min, supernatants were transferred into a pre-weighed scintillation vial, and dried under nitrogen.

5.2.4 Peroxide value determination

Peroxide values were measured by treating each oil sample (5 ± 0.05 g) prepared in 30 ml acetic acid-chloroform (3:2, v:v) with 0.5 ml of saturated potassium iodide solution, followed by titration with 0.1 N sodium thiosulphate (Society and Firestone 1994).

5.2.5 Analysis of fatty acid composition

Lipid extracts were dissolved in 2 ml of n-heptane and treated with 0.2 ml of 2 N potassium hydroxide methanolic solution (11,2 g of potassium hydroxide in 100 ml methanol). The mixture was shacked vigorously for 1 min at room temperature and then centrifuged (3000 g for 5 min). Supernatants were collected and analysis of fatty acid methyl esters was performed by gas chromatography using a DANI GC instrument (DANI Instruments, Milan, Italy) coupled to a flame ionization detector (FID) and equipped with a HP-5 capillary column (Agilent, Milan, Italy). The temperature program started at 150 °C (10 min), increased by 2 °C/min to 180 °C and then increased again by 3 °C/min to 240 °C (20 min).

5.2.6 Study population and protocol

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in February 2017. Patients aged 18-83 years were eligible for enrolment if they had the following values of serum parameters at baseline: TC, 200-260 mg/dl; HDL-C, 31-45 mg/dl; LDL-C, 179-205 mg/dl; glucose, 90-125 mg/dl; TG, 170-280 mg/dl. The subjects were asked to keep their dietary habits unchanged throughout the entire study.

Exclusion criteria were: smoking, obesity (BMI > 30 kg/m2), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, drug therapy or supplement intake for hypercholesterolemia, drug therapy or supplement intake containing apple polyphenols, heavy physical exercise (> 10 h/week), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding, birch pollen allergy, use of vitamin/mineral supplements 2 weeks prior to entry into the study and donation of blood less than 3 months before the study.

The subjects received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (protocol 26875 of 22/02/2016) and carried out in accordance with the Helsinki declaration of 1964 (as

revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a randomised, monocentric, placebo-controlled trial conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 16 weeks: the group underwent 4 weeks of placebo treatment, consisting in the administration of identically appearing capsules containing only maltodextrin, followed by 8 weeks of nutraceutical treatment, and 4 weeks of follow-up. Both the examinations and the study treatment were performed in an outpatient setting. Clinic visits and blood sampling were performed after 12 h of fasting at weeks 0, 4, 8, 12, and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10-mL EDTA-coated tubes (Becton–Dickinson, Plymouth, UK) and plasma was isolated by centrifugation (20 min, 2.200 g, 4 °C). All samples were stored at -80 °C until analysis. Plasma TC, HDL-C, LDL-C, glucose, and TG levels were determined using commercially available kits from Diacron International (Grosseto, Italy). Analyses were performed on a Diacron International Free Carpe Diem, and intra- and inter-day variations were 1.4 and 1.6% for TC, 1.6 and 2.2% for LDL-C, 2.0 and 2.3% for HDL-C, 1.1 and 1.7% for glucose, and 1.3 and 1.8% for TG, respectively. In addition to these five meetings, six standardised telephone interviews were performed every 14 days starting from the first meeting, to verify compliance and increase protocol adherence. In particular, these interviews reminded patients to complete their intake-checking table for the intervention study and to record any treatment discontinuation, or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit).

All patients underwent a standardised physical examination, assessment of medical history (for up to five years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. At each clinic visit, patients had to complete three self-administered questionnaires on quality of life aspects, and their diaries were checked for data completeness and quality of documentation to ensure patient comprehension of the diary items.

5.2.7 Randomisation, concealment, and blinding

A total of 150 eligible patients were randomly assigned to five sub-groups. If a patient dropped out before the intervention period, he or she was replaced by the next eligible

patient enrolled at the same centre. The concealed allocation was performed by an internet-based randomisation schedule, stratified by study site. The random number list was generated by an investigator with no clinical involvement in the trial. Patients, clinicians, core laboratories, and trial staff (data analysts, statisticians) were blind to treatment allocation.

5.2.8 Study treatments

The group of 150 patients (80 men and 70 women, 18-83 years of age) was randomly divided into five subgroups (each one of 30 subjects, 16 men and 14 women). The volunteers enrolled in this study had the following values of plasma parameters at baseline: TC, 200-260 mg/dl; HDL-C, 31-45 mg/dl; LDL-C, 179-205 mg/dl; glucose, 90-125 mg/dl; TG, 170-280 mg/dl. Each sub-group was assigned to a different intervention, as follows: sub-group 1, 5 g chia seeds/day; sub-group 2, 5 g cryo-micronized chia seeds/day; sub-group 3, 4 GR capsules containing cryo-micronized chia seeds (500 mg each)/day; sub-group 4, 4 GR capsules containing cryo-micronized chia seeds (500 mg each) and vitamin E (15 mg each)/day; sub-group 5, 4 GR capsules containing vitamin E (15 mg each)/day. All subjects assuming capsules were instructed to take two capsules at lunch, and two capsules at dinner.

5.2.9 Study outcomes and data collection

5.2.9.1 Primary and secondary efficacy outcomes

Primary endpoints measured were the variations of TC, HDL-C, LDL-C, glucose, and TG, while key secondary outcomes collected during clinic visits were measurements of blood pressure and heart rate, and evaluation of BMI.

All raw patient ratings were evaluated in a blinded manner at the site of the principal investigator. The decision process was performed according to a consensus document (unpublished standard operating procedure) before unblinding in order to define conclusive primary and secondary efficacy data from a clinical perspective.

5.2.9.2 Safety

We assessed safety from reports of adverse events as well as laboratory parameters concerning the hepatic and renal function, vital signs (blood pressure, pulse, height, weight, and body mass index), and physical or neurological examinations. Safety was assessed over the entire treatment period at weeks 0, 4, 8, and 12, including adverse events occurring in the first three weeks after cessation of treatments.

5.2.10 Statistics

5.2.10.1 Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) (Little and Rubin 2019), but also provides reasonably stable results even when the assumption of MAR is violated (Molenberghs, et al. 2004; O'Kelly and Ratitch 2014). Patients who did not provide any diary data (leading to zero evaluable days) were excluded from the MAR based primary efficacy analysis, according to an "all observed data approach" as proposed by White, et al. (2012). This approach is statistically efficient without using multiple imputation techniques (Carpenter and Kenward 2007). Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed by using Cochran's test ($p<0.1$). The I^2 statistic was also calculated, and $I^2>50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percent change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, p-values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration, total polyphenols dose, and Jadad score. Treatment effects were analysed using PROC MIXED with treatment and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum parameters and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P < 0.05$).

5.2.10.2 Analysis sets

The full analysis set population included all randomised patients, and patients who did not fail to satisfy a major entry criterion. We excluded patients who provided neither primary nor secondary efficacy data from efficacy analyses. The per protocol set consisted of all patients who did not substantially deviate from the protocol; they had two characteristics. Firstly, this group included patients for whom no major protocol violations were detected (for example, poor compliance, errors in treatment assignment). Secondly, they had to have been on treatment for at least 50 days counting from day of first intake (completion of a certain pre-specified minimal exposure to the treatment regimen). Hence, patients who prematurely discontinued the study or treatment before day 44 were excluded from the per protocol sample.

5.2.10.3 Determination of sample size

Sample size calculation was based on an anticipated 30% (0.45 mmol/l) change in plasma TGs (primary outcome) with TG standard deviation 0.387 mmol/l, level of significance 0.05 and 80% power (Calvert, et al. 2013). Thus, a minimum of thirteen subjects had to be recruited in each group.

5.2.11 *Patient involvement*

No patients were involved in setting the research question or the outcome measures, nor were they involved in developing plans for participant recruitment, or the design and implementation of the study. There are no plans to explicitly involve patients in dissemination. Final results will be sent to all participating sites.

5.3 Results

5.3.1 *In vitro GI digestion of chia seeds and chia seed-based formulations: fatty acid bioaccessibility and peroxide values in the intestinal solutions*

Table 5 shows results regarding the effects of the simulated GI digestion on the intestinal fatty acid bioaccessibility of chia seeds (sample 1), cryo-micronized chia seeds (sample 2), GR capsules containing cryo-micronized chia seeds (sample 3), GR capsules containing cryo-micronized chia seeds and vitamin E (sample 4). On average, the following recoveries of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), from each sample, in the intestinal solutions, were detected: sample 1, SFA 1.5%, MUFA and PUFA 0.06%, respectively; sample 2, SFA 50%, MUFA and PUFA 7%, respectively; sample 3, SFA 50%, MUFA and PUFA 20%, respectively; sample 4, SFA 50%, MUFA and PUFA 40%, respectively. Peroxide values measured in

the intestinal solutions after GI digestion of samples 1-4 (Table 6) indicated that the highest unsaturated FA oxidative degradation regards sample 2, followed by sample 1 and sample 3, while the lowest value is shown by sample 4.0

Table 5. *Lipid content and fatty acid composition of chia seeds and gastrointestinal digested chia seed-based samples*

	Control	Intestinal digesta			
Fatty acids	Chia seeds	Sample 1	Sample 2	Sample 3	Sample 4
14:0	0.71 ± 0.01^{a}	0.01 ± 0.007 ^{a}	0.34 ± 0.08 ^{a}	0.29 ± 0.02^{a}	0.30 ± 0.06^{a}
15:0	0.62 ± 0.03^{a}	0.01 ± 0.005 ^{a}	0.29 ± 0.04 ^{a}	0.32 ± 0.02^{a}	0.31 ± 0.04^{a}
16:0	28.6 ± 1.5 ^{b}	0.42 ± 0.03 ^{b}	16.2 ± 1.6 ^{b}	15.9 ± 1.4 ^{b}	16.4 ± 1.8 ^{b}
16:1	0.53 ± 0.04^{a}	nd	0.04± 0.007^{c}	0.16 ± 0.02^{c}	0.25 ± 0.05^{c}
17:0	0.94 ± 0.02^{c}	0.01 ± 0.007 ^{a}	0.50 ± 0.01 ^{d}	0.56 ± 0.01^{d}	0.60 ± 0.09^{d}
17:1	0.54 ± 0.01^{a}	nd	0.04 ± 0.005^{c}	0.18 ± 0.01^{c}	0.24 ± 0.07^{c}
18:0	8.71 ± 0.8 ^{d}	0.12 ± 0.04 ^{c}	5.02 ± 0.7 ^{e}	4.68 ± 0.2 ^{e}	4.97 ± 0.10^{e}
18:1	30.1 ± 1.7 ^{b}	0.02 ± 0.006 ^{a}	2.06 ± 0.1 ^{f}	6.34 ± 0.9 ^{f}	11.9 ± 1.2 ^{f}
18:2 ω-6	60.2 ± 1.9 ^{d}	0.06 ± 0.01 ^{d}	4.45 ± 0.2 ^{e}	11.8 ± 1.1 ^{g}	25.7 ± 2.1 ^{g}
20:0	1.72 ± 0.2 ^{a}	0.02 ± 0.007 ^{a}	0.92 ± 0.01 ^{g}	0.86 ± 0.06^{h}	0.77 ± 0.06^{d}
18:3 ω-6	1.33 ± 0.2 ^{a}	0.002 ± 0.0004^{e}	0.07 ± 0.001^{c}	0.32 ± 0.01^{a}	0.59 ± 0.02^{d}
18:3 ω-3	232.1 ± 5.9^{e}	0.26 ± 0.09 ^{e}	15.8 ± 2.5 ^{b}	44.1 ± 3.1 ^{i}	94.8 ± 4.2 ^{h}
20:1	0.60 ± 0.03^{a}	nd	0.05 ± 0.008^{c}	0.12 ± 0.01^{c}	0.31± 0.06^{a}
20:2 ω-6	0.42 ± 0.02^{a}	nd	0.02± 0.007^{c}	0.13 ± 0.02^{c}	0.34 ± 0.04^{a}
22:0	0.51 ± 0.03^{a}	0.01 ± 0.004 ^{a}	0.22 ± 0.06 ^{h}	0.19 ± 0.02^{c}	0.23 ± 0.02^{c}
24:0	0.30 ± 0.01^{a}	0.007 ± 0.001 ^{f}	0.17 ± 0.07 ^{h}	0.20 ± 0.01^{c}	0.22 ± 0.03^{c}
Groups					
SFA	42.0 ± 1.9 ^{f}	0.06 ± 0.001 ^{g}	23.7 ± 1.8 ^{i}	23.0 ± 2.1 ^{h}	23.8 ± 2.4 ^{g}
MUFA	31.7 ± 1.8 ^{b}	0.04 ± 0.005 ^{g}	2.19 ± 0.6 ^{f}	6.80 ± 0.9 ^{f}	12.7 ± 2.1 ^{f}
PUFA	244.0 ± 6.9^{e}	0.44 ± 0.08 ^{b}	20.3 ± 2.2 ^{i}	56.3 ± 5.2 ^{j}	121.4 ± 4.8^{i}
Ratio ω-3/ω-6	3.74	4.33	3.48	3.60	3.56
Lipids	317.7 ± 8.4^{g}	0.54 ± 0.11 ^{b}	46.2 ± 3.1 ^{j}	86.1 ± 1.31^{k}	157.9 ± 4.6^{j}

Values are expressed as mg fatty acids/g chia seeds and are reported as mean ± SD of five replicate analyses ($n = 5$).
[abcdefghijk] Mean values in columns with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
Sample 1: 500 mg chia seeds; Sample 2, 500 mg cryo-micronized chia seeds; Sample 3, 1 gastric-resistant capsule containing cryo-micronized chia seeds (500 mg); Sample 4, 1 gastric-resistant capsule containing cryo-micronized chia seeds (500 mg) and vitamin E (15 mg).
nd: not detected.

Table 6. *Peroxide values of intestinal solutions after gastrointestinal digestion of chia seeds and chia seed-based samples*

	Intestinal digesta			
	Sample 1	Sample 2	Sample 3	Sample 4
Peroxide value (meq O_2/kg)	0.18 ± 0.02 ^{a}	0.48 ± 0.05 ^{b}	0.16 ± 0.03 ^{a}	0.035 ± 0.002 ^{c}

Values are reported as mean ± SD of five replicate analyses ($n = 5$).
[abc]Mean values in raw with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).
Sample 1: 500 mg chia seeds; Sample 2, 500 mg cryo-micronized chia seeds; Sample 3, 1 gastric-resistant capsule containing cryo-micronized chia seeds (500 mg); Sample 4, 1 gastric-resistant capsule containing cryo-micronized chia seeds (500 mg) and vitamin E (15 mg).

5.3.2 *Enrolment and subject attrition*

Patients were enrolled in February 2017. A total of 204 patients were screened for eligibility; 54 patients (26.5%) did not pass the screening stage; 150 patients were randomised. The most common reason was that patients did not meet the inclusion criteria regarding values of serum parameters at baseline (n=23), followed by general refusal to participate for no specific reasons (n=8), and concerns about the protocol, especially fear of placebo (n=3). Some fulfilled exclusion criteria (n=20).

Overall, 150 patients were assigned to the group of intervention study: they were divided into five subgroups (each one made of 30 patients). Patients of subgroups underwent a placebo period during the first 4 weeks before the treatment period of 8 weeks. Follow-up period last other 4 weeks. Figure 7 shows the flow of participants through the trials

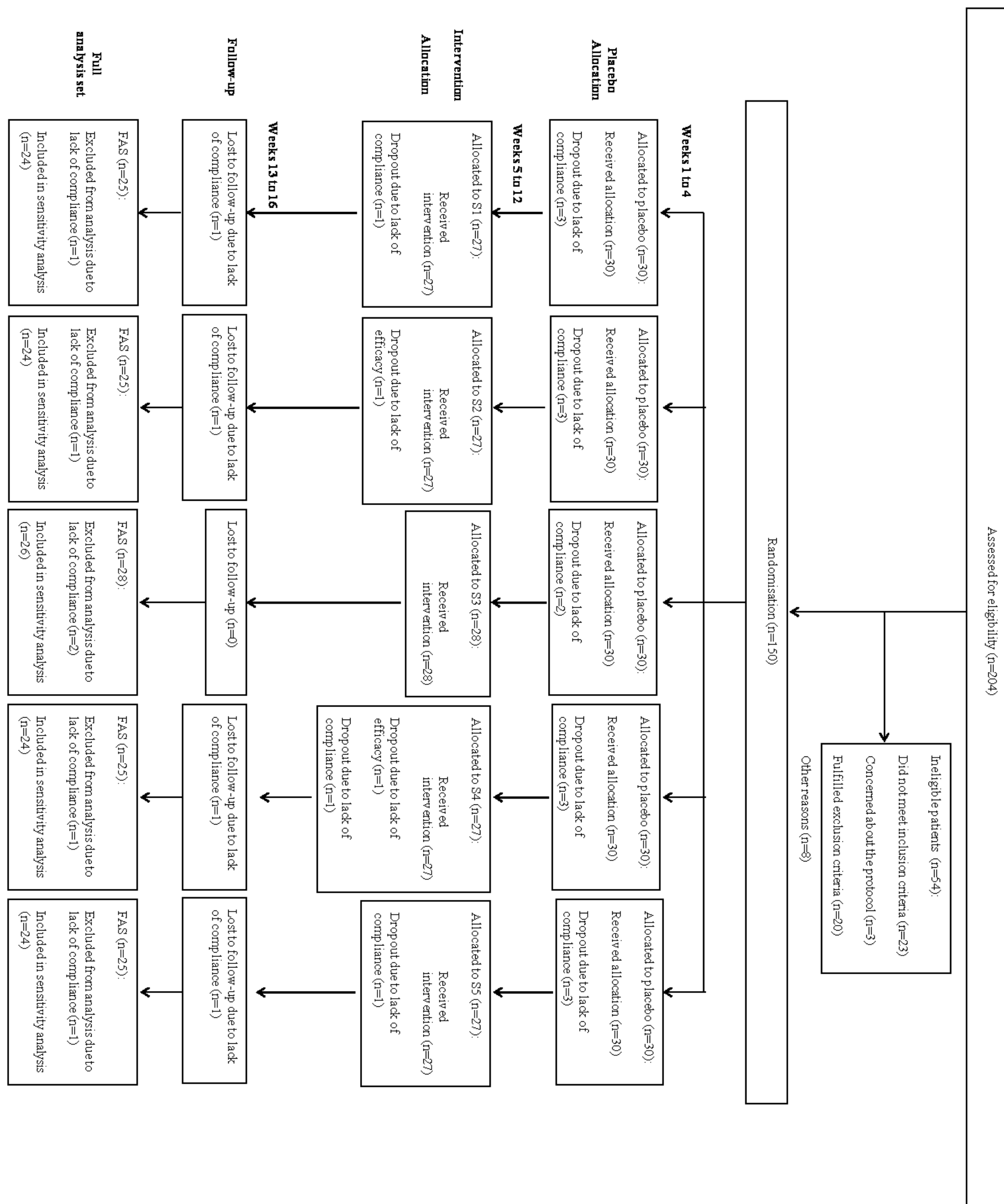

Figure 7. Study flowchart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from prescreening to data collection; and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period. S1 (subgroup 1)=Sample 1 (chia seeds); S2 (subgroup 2)=Sample 2 (cryo-micronized chia seeds); S3 (subgroup 3)=Sample 3 (gastric-resistant capsules containing cryo-micronized chia seeds); S4 (subgroup 4)=Sample 4 (gastric-resistant capsules containing cryo-micronized chia seeds and vitamin E); S5 (subgroup 5)=Sample 5 (gastric-resistant capsules containing vitamin E); FAS=full analysis set.

together with the completeness of diary information over the entire treatment period.

No patient prematurely terminated study participation before allocation to treatment. Figure 7 follows the CONSORT PRO reporting guideline (Raederstorff, et al. 2015) and reveals that within the assessment period, the following percentage of patients for each subgroup provided data for the primary endpoint: subgroup 1, 88.9% (24 of 27 patients); subgroup 2, 88.9% (24 of 27 patients); subgroup 3, 92.8% (26 of 28 patients); subgroup 4, 88.9% (24 of 27 patients); subgroup 5, 88.9% (24 of 27 patients). In each group, a few patients did not submit any diaries, giving no specific reason for this. Completeness of the patient diaries did not differ between the treatment groups.

5.3.3 Participants' baseline characteristics

Table 7 shows the demographic and clinical characteristics assessed at the baseline visit of all 150 patients randomised. Overall, about half of the randomised patients were female; the total age range was 18-83 years. The groups were well balanced for demographics and clinical factors.

Table 7. *Baseline characteristics of intention to treat sample according to study treatment.*

			Placebo		
Characteristics	**S1 (n=30)**	**S2 (n=30)**	**S3 (n=30)**	**S4 (n=30)**	**S5 (n=30)**
Demographics					
Age (years)	43.8 ± 10.5	42.7 ± 11.2	42.4 ± 10.6	44.8 ± 11.2	41.8 ± 10.8
Male sex (No (%))	16 (53.3%)	16 (53.3%)	17 (56.7%)	15 (50.0%)	16 (53.3%)
White ethnicity (No (%))	30 (100%)	30 (100%)	30 (100%)	30 (100%)	30 (100%)
Clinical parameters					
TC (mg/dl)	239.1 ± 13.4	233.5 ± 12.6	240.2 ± 11.5	236.3 ± 12.4	236.1 ± 13.8
LDL-C (mg/dl)	191.0 ± 11.3	184.1 ± 12.3	187.9 ± 12.1	185.2 ± 11.9	187.8 ± 13.1
HDL-C (mg/dl)	36.8 ± 6.1	36.3 ± 7.2	39.9 ± 6.8	36.2 ± 7.7	32.9 ± 7.3
Glucose (mg/dl)	99.5 ± 8.1	91.4 ± 9.4	111.2 ± 9.2	100.8 ± 2.2	100.1 ± 7.3
Triglycerides (mg/dl)	187.1 ± 9.2	222.8 ± 12.1	193.6 ± 11.8	202.4 ± 10.2	212.3 ± 12.1
			Treatment		
Characteristics	**S1 (n=27)**	**S2 (n=27)**	**S3 (n=28)**	**S4 (n=27)**	**S5 (n=27)**
Demographics					
Age (years)	44.2 ± 10.6	45.5 ± 10.7	43.5 ± 10.2	42.1 ± 11.1	43.8 ± 12.2
Male sex (No (%))	15 (55.5%)	14 (51.8%)	13 (46.4%)	14 (51.8%)	15 (55.5%)
White ethnicity (No (%))	27 (100%)	27 (100%)	27 (100%)	27 (100%)	27 (100%)
Clinical parameters					
TC (mg/dl)	235.5 ± 13.3	237.6 ± 14.3	239.1 ± 11.9	235.9 ± 13.7	238.4 ± 15.3

LDL-C (mg/dl)	185.7 ± 11.1	189.1 ± 11.6	189.6 ± 11.7	183.7 ± 12.3	189.1 ± 11.6
HDL-C (mg/dl)	36.7 ± 7.5	35.4 ± 6.7	38.5 ± 7.2	37.9 ± 8.1	34.2 ± 6.9
Glucose (mg/dl)	92.8 ± 2.7	102.1 ± 8.9	108.2 ± 8.5	101.9 ± 10.0	99.5 ± 9.3
Triglycerides (mg/dl)	220.0 ± 16.8	185.4 ± 8.3	195.2 ± 9.0	206.1 ± 10.4	210.5 ± 11.8

Values are means ± SD (n = 5).
Results were significantly different at a level of P = 0.001.
S1 (subgroup 1)=Sample 1 (chia seeds); S2 (subgroup 2)=Sample 2 (cryo-micronized chia seeds); S3 (subgroup 3)=Sample 3 (gastric-resistant capsules containing cryo-micronized chia seeds); S4 (subgroup 4)=Sample 4 (gastric-resistant capsules containing cryo-micronized chia seeds and vitamin E); S5 (subgroup 5)=Sample 5 (gastric-resistant capsules containing vitamin E).

5.3.4 Primary efficacy outcome measures

No significant variation of plasma TC, LDL-C, HDL-C, glucose, and TG levels, with respect to the baseline values, was registered at the end of placebo period in subjects of all of subgroups. Analysing results, we can assert that the administration of all of samples 1-5 led, in all subgroups, to a statistically significant variation of plasma TC, LDL-C, HDL-C, glucose, and TG levels (Table 8). On balance, the most important variation was registered by all of samples for TG levels (Table 8). Specifically, at the end of the intervention period, sample 4 exerted the highest TG lowering effect, decreasing TG by 27.5% (P = 0.0095), followed by sample 3 and sample 2, which led the TG levels to -17.5% (P = 0.0042) and -10.2% (P = 0.0032), respectively, while sample 1 and sample 5 showed the lowest effects (-6.3%, P = 0.0036, and -3.1%, P = 0.0029, respectively). Analysing the TC values in patients supplied with the different samples, we can assert that sample 4 was approximately 1.5 folds more effective in reducing the TC levels (-8.0 %, P = 0.0019) than sample 3 (-5.6%, P = 0.0012) which was the second most active among all the samples tested. The same trend was maintained as regards the LDL-C levels. Interestingly, HDL-C levels significantly raised in patients belonging to all subgroups. Once again, sample 4 showed the greatest effect (+5.7 %, *P* = 0.0042) with respect to sample 3 (+3.4%, *P* = 0.0027), sample 2 (+1.8%, P = 0.0095), sample 1 (+1.4%, P = 0.0042), and sample 5 (+1.3%, and -10.2% (P = 0.0032). A slight hypoglycaemic effect was revealed in all subjects belonging to all subgroups (Table 8). Noteworthy, all of the experimental results were achieved already after one month of intervention study and were confirmed at the end of the second month. Comparing the *in vitro* results regarding the effect of simulated GI digestion on bioaccessibility of fatty acids in chia seed and chia seed-based formulations (Table 5), with the clinical value concerning the TG lowering effects of samples 1-5 (Table 8), a significant correlation was found. Specifically, sample 4, exhibiting the highest bioaccessible ω-3 FA fraction (Table 5), was the most effective in decreasing human TG

levels (Table 8), while samples 1 and 2, characterised by the *in vitro* least bioaccessible ω-3 FA fraction, showed the poorest effects.

Table 8. Effects of chia seeds and chia seed based formulations on plasma cholesterol, glucose and triglyceride metabolism.

		S1	Δ(%)	S2	Δ(%)	S3	Δ(%)	S4	Δ(%)	S5	Δ(%)
TC (mg/dL)	*t0*	235.5±13.3		237.6±14.3		239.1±11.9		235.9±13.7		238.4±15.3	
	t30	231.3±14.2	*-1.8*	229.7±12.8	*-3.3*	226.2±13.5	*-5.4*	216.3±14.1	*-8.3*	235.3±11.9	*-1.3*
	t60	230.3±13.6	*-2.2*	229.5±12.1	*-3.4*	225.7±13.8	*-5.6*	217.0±13.4	*-8.0*	235.8±12.0	*-1.1*
LDL-C (mg/dL)	*t0*	185.7±11.1		189.1±11.6		189.6±11.7		183.7±12.3		189.1±11.6	
	t30	181.8±11.3	*-2.1*	182.3±10.9	*-3.6*	177.6±0.8	*-6.3*	166.1±11.7	*-9.6*	184.1±12.0	*-2.6*
	t60	182.2±13.6	*-1.9*	181.5±11.2	*-4.0*	178.2±1.2	*-6.0*	165.0±12.1	*-10.2*	184.1±12.3	*-2.6*
HDL-C (mg/dL)	*t0*	36.7±7.5		35.4±6.7		38.5±7.2		37.9±8.1		34.2±6.9	
	t30	37.3±7.5	*+1.6*	36.2±7.9	*+2.2*	40.0±8.0	*+3.8*	40.1±6.8	*+5.9*	34.7±6.4	*+1.5*
	t60	37.2±7.0	*+1.4*	36.0±8.1	*+1.8*	39.8±8.3	*+3.4*	40.1±6.0	*+5.7*	34.6±5.6	*+1.3*
Glucose (mg/dL)	*t0*	92.8±12.7		102.1±8.9		108.2±8.5		101.9±10.0		99.5±9.3	
	t30	92.1±10.8	*-0.7*	101.0±12.0	*-1.1*	107.5±12.4	*-2.1*	98.8±8.3	*-3.0*	98.4±10.1	*-1.1*
	t60	91.9±11.6	*-1.0*	100.3±13.1	*-1.8*	105.5±10.3	*-2.5*	98.6±10.1	*-3.2*	98.2±19.8	*-1.3*
Triglycerides (mg/dL)	*t0*	220.0±16.8		185.4±8.3		195.2±19.0		206.1±10.4		210.5±11.8	
	t30	187.5±19.3	*-5.7*	167.4±16.4	*-9.7*	160.4±21.1	*-17.8*	152.9±16.7	*-25.8*	203.1±13.4	*-3.5*
	t60	186.1±14.7	*-6.3*	166.5±17.3	*-10.2*	161.0±15.2	*-17.5*	149.4±14.2	*-27.5*	204.0±16.5	*-3.1*

Values are means ± SD (n = 5); results were significantly different at a level of P = 0.001.
S1 (subgroup 1)=Sample 1 (chia seeds);
S2 (subgroup 2)=Sample 2 (cryo-micronized chia seeds);
S3 (subgroup 3)=Sample 3 (gastric-resistant capsules containing cryo-micronized chia seeds);
S4 (subgroup 4)=Sample 4 (gastric-resistant capsules containing cryo-micronized chia seeds and vitamin E);
S5 (subgroup 5)=Sample 5 (gastric-resistant capsules containing vitamin E).

5.3.5 *Safety issue, study strength and limitations*

Table 9 shows data regarding blood indicators of potential hepatic and renal functions. Clinical results clearly indicated that no toxicity was registered during and at the end of the intervention period, except for subjects assuming cryo-micronized chia seeds (sample 2) who revealed an average slight increase of all of measured parameters. Other safety assessments, such as vital signs, blood pressure, or electrocardiographic findings, were all periodically monitored, and baseline values did not change substantially during and at the end of the trial.

The major strengths of the clinical trial herein presented reside in the originality of the study and in the evaluation of the treatment effects in real-world settings. Conversely, the main limitations of our study include the short-term assessment for the treatment of a chronic condition and the choice of exclusively white race.

Table 9. Effects of chia seeds and chia seed based formulations on plasma indicators of hepatic and renal function.

		S1	Δ (%)	S2	Δ (%)	S3	Δ (%)	S4	Δ (%)	S5	Δ (%)
AST (GOT) (U/L)	*t0*	30.2 ± 7.2		25.3 ± 6.7		34.1 ± 7.6		21.6 ± 5.4		38.2 ± 7.5	
	t30	29.1 ± 5.3	*-3.6*	26.6 ± 4.8	*+5.2*	34.9 ± 6.8	*+2.5*	21.5 ± 4.8	*-0.5*	37.7 ± 8.1	*-1.3*
	t60	29.4 ± 7.9	*-2.6*	26.8 ± 5.9	*+6.0*	35.1 ± 8.1	*+3.1*	21.5 ± 5.2	*-0.5*	37.8 ± 6.8	*-1.1*
ALT (GPT) (U/L)	*t0*	22.6 ± 6.4		34.7 ± 8.2		30.4 ± 5.4		27.9 ± 3.8		25.6 ± 4.7	
	t30	22.0 ± 5.8	*-2.6*	35.4 ± 7.5	*+2.1*	30.7 ± 5.9	*+1.1*	27.5 ± 4.2	*-1.4*	25.4 ± 5.1	*-2.6*
	t60	22.1 ± 4.9	*-2.2*	35.7 ± 6.9	*+2.9*	30.9 ± 6.9	*+1.7*	27.6 ± 4.9	*-1.1*	25.4 ± 4.6	*-2.6*
γ-GTP (U/L)	*t0*	28.9 ± 6.2		32.8 ± 5.5		20.7 ± 3.8		37.2 ± 5.8		30.5 ± 5.8	
	t30	28.4 ± 8.2	*-1.7*	33.9 ± 5.3	*+3.5*	21.0 ± 4.2	*+1.6*	36.2 ± 6.3	*-2.7*	30.9 ± 5.1	*+1.5*
	t60	28.3 ± 7.1	*-2.1*	33.8 ± 6.2	*+3.2*	20.9 ± 6.3	*+1.4*	34.4 ± 5.9	*-7.5*	30.8 ± 4.8	*+1.3*
ALP (U/L)	*t0*	103.2 ± 11.6		89.5 ± 9.2		77.4 ± 8.9		100.2 ± 9.7		80.2 ± 6.3	
	t30	102.7 ± 10.5	*-0.48*	91.5 ± 8.7	*+2.3*	78.2 ± 9.6	*+1.0*	99.4 ± 10.6	*-0.9*	79.3 ± 7.2	*-1.1*
	t60	102.5 ± 10.4	*-0.68*	91.1 ± 8.8	*+1.8*	77.9 ± 7.5	*+0.7*	99.1 ± 8.6	*-1.1*	79.1 ± 6.9	*-1.3*
LDH (U/L)	*t0*	165.9 ± 12.3		189.2 ± 10.1		195.2 ± 13.7		177.0 ± 11.6		200.1 ± 14.7	
	t30	165.0 ± 11.8	*-0.54*	188.2 ± 11.1	*-0.5*	193.0 ± 12.8	*-1.1*	176.5 ± 12.8	*-0.3*	193.1 ± 13.6	*-3.5*
	t60	164.8 ± 13.9	*-0.66*	188.1 ± 10.4	*-0.6*	192.5 ± 14.8	*-1.4*	170.2 ± 10.7	*-3.8*	193.9 ± 13.2	*-3.1*
Albumin (g/dL)	*t0*	4.84 ± 0.97		4.25 ± 0.88		4.06 ± 0.92		4.31 ± 0.83		3.98 ± 0.77	
	t30	4.62 ± 0.85	*-4.5*	4.38 ± 0.79	*+3.1*	4.12 ± 0.88	*+1.6*	4.32 ± 0.86	*+0.2*	4.03 ± 0.85	*+1.5*
	t60	4.46 ± 0.79	*-7.8*	4.42 ± 0.83	*+4.1*	4.16 ± 0.85	*+2.4*	4.16 ± 0.91	*-3.5*	4.01 ± 0.89	*+1.0*
Total bilirubin (mg/dL)	*t0*	0.66 ± 0.11		0.82 ± 0.07		0.48 ± 0.05		0.57 ± 0.08		0.78 ± 0.07	
	t30	0.60 ± 0.10	*-9.0*	0.85 ± 0.08	*+3.4*	0.49 ± 0.06	*+1.8*	0.57 ± 0.09	-	0.76 ± 0.07	*-2.4*
	t60	0.62 ± 0.12	*-6.0*	0.86 ± 0.09	*+4.4*	0.50 ± 0.07	*+2.3*	0.54 ± 0.05	*-5.3*	0.75 ± 0.09	*-2.2*
Creatinine (mg/dL)	*t0*	0.88 ± 0.09		1.04 ± 0.12		0.97 ± 0.09		0.84 ± 0.11		1.12 ± 0.12	
	t30	0.87 ± 0.10	*-1.1*	1.07 ± 0.13	*+2.7*	0.99 ± 0.08	*+1.7*	0.82 ± 0.09	*-2.4*	1.11 ± 0.11	*-0.9*
	t60	0.89 ± 0.08	*+1.1*	1.08 ± 0.16	*+3.2*	0.98 ± 0.07	*+1.5*	0.80 ± 0.12	*-4.8*	1.10 ± 0.12	*-0.7*

Values are means ± SD (n = 5); results were significantly different at a level of P = 0.001.

S1 (subgroup 1)=Sample 1 (chia seeds); S2 (subgroup 2)=Sample 2 (cryo-micronized chia seeds); S3 (subgroup 3)=Sample 3 (gastric-resistant capsules containing cryo-micronized chia seeds);

S4 (subgroup 4)=Sample 4 (gastric-resistant capsules containing cryo-micronized chia seeds and vitamin E); S5 (subgroup 5)=Sample 5 (gastric-resistant capsules containing vitamin E).

5.4 Discussions

Our *in vitro* experimental results (Table 5) clearly indicated that chia seeds, although their high amount of PUFA (24.4%) and, specifically, of ω-3 FA (23.2%), are characterised by a quite low intestinal bioaccessibility of such bioactive compounds. Thus, the extensively reported healthy effects of chia seeds, referred to their important PUFA content, seem to remain just a potential beneficial property, not realistically achievable by their regular consumption. In support to these data, our results from the clinical study would confirm that, after two months of regular consumption, chia seeds are only able of a modest beneficial influence on blood TGs (-6.3%), as well as cholesterol and glucose levels (Table 8). It could be hypothesised that, due to their very small size, and their resistance to mastication, chia seeds would be very difficult, or almost impossible, to be properly chewed and, thus, crushed, so to make their PUFA content quite low or not bioaccessible.

Interestingly, micronized chia seeds, although strongly improving SFA intestinal bioaccessibility (about +50%), only provided a modest increase of MUFA and PUFA release (about +7%, respectively), when submitted to simulated GI digestion (Table 5). Such *in vitro* observation would find significant substantiation in our clinical study which highlights a slight increase in efficacy on metabolic parameters in subjects assuming micronized chia seeds (Table 8). The quite low MUFA and PUFA intestinal bioaccessibility could be ascribed to their massive oxidative degradation, as indicated by our *in vitro* measures of lipid peroxidation index in the intestinal solution after GI digestion of micronized chia seeds (Table 6).

Actually, it is well known that the effects of GI digestion on vegetable and animal oils, not only lead to a significant degradation of unsaturated FA, thus, much decreasing their actual intestinal bioaccessibility (Cofrades, et al. 2017; Domoto, et al. 2013; Nieva-Echevarría, et al. 2017), but, more importantly, also favour the development of oxidation products which would be absorbed along the duodenal tract (Maestre, et al. 2013).

Thus, we decided to include micronized chia seeds in GR capsules and to submit them to simulated GI digestion. As expected, SFA were still available in the intestinal layer at the same level (about +50%) (Table 1), confirming that these FA are released after seed micronization and are quite resistant to gastric environment. Interestingly, MUFA and PUFA much increased their bioaccessibility (+20%) (Table 5). This could be due to their protection against the gastric degradation, as confirmed by our peroxide index measures (Table 6), which clearly indicated an important decrease of unsaturated FA oxidative degradation, compared to non gastro-protected micronized chia seeds. To confirm these

results, our clinical data from subjects assuming GR capsules containing micronized chia seeds (Table 8), highlighted an evident correlation with *in vitro* digestion measures, and, again, the most important clinical effect regarded the decrease of blood TG levels (on average, -17.5%).

Considering the significant decrease of unsaturated FA peroxidation, regarding the gastro-protected micronized chia seeds (Table 6), we have hypothesised to further improve the protection against lipoperoxidative degradation of such formulation by adding a specific lipophilic antioxidant compound. The choice of vitamin E has revealed successful since it has doubled the MUFA and PUFA *in vitro* intestinal bioaccessibility (+40%) (Table 5), probably due to its important protection of unsaturated FA against oxidative degradation (Raederstorff, et al. 2015), as clearly indicated by our results regarding the drastically reduced peroxide values (Table 6). Such fortified formulation, administered to subjects under the dosage of four capsules/day for two months, lowered blood TG levels by about 27.5%, that means a +10% efficacy respect to the chia seed-based GR formulation non including vitamin E (Table 8). Interestingly, a peculiar aspect concerning both GR formulations regards the evident synergistic effect obtained by the sum of the individual components (capsules and contents) in providing the clinical results. In fact, a total dosage of 2 g chia seeds/day, provided by 4 capsules of both formulations, allowed a much higher decrease of blood TG levels than what obtained by assuming 5 g of either chia seeds or micronized chia seeds/day. Particularly, results were as follows: GR capsules containing micronized chia seeds, 2.8 folds chia seeds, 1.7 folds micronized chia seeds; GR capsules containing micronized chia seeds and vitamin E, 4.4 folds chia seeds, 2.7 folds micronized chia seeds. The very modest effects on all of blood parameters obtained by administering subjects only the gastro-protected vitamin E (Table 8), further corroborates the synergistic importance of the different components included in the formulation.

Although no specific toxicity studies have been performed herein, the type and daily quantity of components assumed by subjects of each group are expected to be totally compatible with a healthy state. In fact, chia seeds have been used at a maximum quantity of 5 g/day, which is lower than the daily amount suggested as common nuts for a cardioprotective potential (about 30 g). Vitamin E (60 g/day) is in full accordance with the maximum quantity allowed in food supplements for a daily intake (Reg. EU 1169/2011). To confirm this, our clinical data regarding the measures of hepatic and renal function (Table 9) clearly indicate no signs of toxicity. An exception would be represented by subjects who assumed non-GR cryo-micronized chia seeds, revealing an average slight

increase of all of measured parameters. Probably, the highest peroxidative degradation of unsaturated fatty acids, highlighted by our *in vitro* experiment of GI digestion, in the intestinal solution (Table 6), could suggest for subjects assuming non-GR cryo-micronized chia seeds a significant production of lipoperoxides, which would be absorbed along the duodenal tract, with potential toxicological implications, as already demonstrated for vegetable and animal oils by other authors.

5.5 Conclusions

The present study proposes an innovative nutraceutical product which is able to address all of these conditions. Particularly, this formulation is characterised by an evident synergistic effect of all of its components, since its bioactive principle can provide more than four times higher therapeutic effect than what offered by less than the half quantity of the same bioactive principle not included in the formulation. Moreover, the typical potential toxic effects of the original food seem to be almost totally deleted. Our nutraceutical formulation can be regarded as an effective and safe natural remedy for the contribution to a healthy balance of plasma TG levels.

6 Bioactive peptide derived from gastrointestinal digestion of "Mozzarella di Bufala Campana PDO": chemical characterization, bioavailability, *in vitro* antioxidant activity and intestinal protection

6.1 Introduction

In addition to its major nutritional role, the bioactive potential of milk is now well accepted. It has been recognised that much of the bioactivity can be attributed not only to intact caseins and whey proteins but also to many milk protein-derived peptides (Korhonen 2009). Such peptides are inactive within the sequence of the parent protein and can be released by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing (Kitts and Weiler 2003). They can have a beneficial effect on a variety of biological systems including the cardiovascular, gastrointestinal, immune and nervous systems (Murray and FitzGerald 2007).

Reactive oxygen species (ROS), the by-products of oxygen metabolism, are among the main causes of a wide range of human degenerative pathologies, such as cardiovascular diseases, diabetes, inflammation, cancer, neurodegeneration, and accelerated aging, by a process named oxidative stress (Di Bernardini, et al. 2011; Mendis, et al. 2007). Peptides contribute to the body antioxidant protection, in particular they can on average scavenge 20% (range 10–50 %) of the peroxyl radicals of plasma (Wayner, et al. 1987). Several milk-derived peptides have been found to possess antioxidant activity (Pihlanto 2006). Particularly, caseinphosphopeptides (CPPs) derived from enzymatic hydrolysis of casein and are rich in phosphoserine residues. The proposed mechanism of CPP antioxidant activity is linked to the presence of phosphate groups originating from the phosphoserine residues in close proximity to the peptide chain (Kitts 2005). CPPs have been shown to possess radical scavenging (Chiu and Kitts 2004) and metal chelating activity (Kim, et al. 2007), whereas high amounts of CPP have been reported to be pro-oxidative (Díaz and Decker 2004).

Peptides can be absorbed in the intestinal tract by a number of mechanisms including the paracellular and transcellular routes, the lymphatic system and via basolateral transporters. The molecular size and structural properties such as peptide hydrophobicity will determine the mechanism of transport (Segura-Campos, et al. 2011). Nevertheless, a bioactive peptide may not need to be absorbed in order to elicit the antioxidant function. Binding to an intestinal receptor may be sufficient to trigger the bioactive (i.e. antioxidant) response (Xiong 2010).

The intestine is at the interface between the organism and its luminal environment, thus, representing a critical defence barrier against luminal toxic agents. Therefore, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated ROS (Aw, 1999). The latter may also elicit a beneficial biological effect by reducing oxidative damage within the gastrointestinal tract (Xiong, 2010).

Buffalo (*Bubalus bubalis*) milk is a source of many bioactive peptides that have been demonstrated to play a relevant role in preventing various disorders (Chanu, et al. 2018; De Simone, et al. 2011; De Simone, et al. 2009; Mohanty, et al. 2016). In particular, ACE-inhibitory and antimicrobial peptides after enzymatic digestion of buffalo milk casein have been described (De Simone, et al. 2011), while buffalo mozzarella cheese whey has been indicated as a major source of antiproliferative and cytomodulatory peptides (De Simone, et al. 2009). Rizzello, et al. (2005) found that the peptides extracted from Italian buffalo mozzarella cheese exhibited a specific antibacterial activity in comparison to peptides from other cheeses. However, no studies on bioactive buffalo mozzarella cheese peptides derived from gastrointestinal digestion are available to date. Thus, the first aim of the present study was to evaluate the peptide production after simulated gastrointestinal digestion of "Mozzarella di Bufala Campana DOP" (MBC) and their potential antioxidant activity by *in vitro* assays.

6.2 Materials and methods

6.2.1 Reagents and standards

All chemicals and reagents used were either analytical-reagent or HPLC grade. Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts, Sephadex G-50, G-25 and G-10, DPPH (1,1-diphenyl-2-picrilhydrazyl), 2,4,6-tris-2,4,6-tripiridyl-S-triazine (TPTZ), iron (III) chloride (dry), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2',7'-dichlorofluorescin diacetate (DCFH-DA), were purchased from Merck Life Science (Milan, Italy). All organic solvents were purchased from Carlo Erba, (Milano, Italy). DCFH-DA was dissolved in dimethyl sulfoxide (DMSO) to obtain 100 mM stock solution (aliquoted and stored at -20°C). Work solutions of DCFH-DA were produced by diluting aliquots in 1% phosphate buffer saline (PBS, 10 mM, pH 7.4) at different concentrations.

6.2.2 *Sample collection and preparation*

"Mozzarella di Bufala Campana DOP" (MBC) was purchased in Caserta (Campania, Italy) in a local dairy. An aliquot (250 g), approximately 1 h after its preparation, was stored at -80 °C. Sample was freeze-dried and, then, subjected to lipid extraction according to AOAC method 948.16 (WINDHAM 1995), by using a 6-place units Extraction Unit E-816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000 g for 5 min, pellets were transferred into a pre-weighed scintillation vial, dried under nitrogen, and kept at -80 °C until analyses.

6.2.3 *In vitro gastrointestinal digestion*

The assay was performed according to the procedure described by Raiola, et al. (2012), with slight modification. GI digestion was distinguished into salivary, gastric and duodenal digestive steps. For the salivary digestion, defatted sample (80.5 g) was mixed with 6 ml of artificial saliva composed of: KCl (89.6 g/l), KSCN (20 g/l), NaH_2PO_4 (88.8 g/l), Na_2SO_4 (57.0 g/l), NaCl (175.3 g/l), $NaHCO_3$ (84.7 g/l), urea (25.0 g/l) and 290 mg of α-amylase. The pH of the solution was adjusted to 6.8 with 0.1 M HCl. The mixture was introduced in a plastic bag containing 40 ml of water and homogenised in a Stomacher 80 Microbiomaster (Seward, Worthing, UK) for 3 min. Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted to 2.0 with 6 M HCl, and then incubated at 37 °C in a Polymax 1040 orbital shaker (250 rpm) (Heidolph, Schwabach, Germany) for 2 h. After the gastric digestion, the pancreatic digestion was simulated as follows: the pH was increased to 6.5 with 0.5 M $NaHCO_3$ and then 5 ml of a mixture pancreatin (8.0 mg/ml) and bile salts (50.0 mg/ml) (1:1; v/v), dissolved in 20 ml of water, were added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. Intestinal digested sample was freeze-dried and stored at -80 °C until further analysis.

6.2.4 *Gel filtration chromatography of MBC gastrointestinal digest*

6.2.4.1 Fraction separation

The freeze-dried intestinal digested sample was dissolved with water (50 mg/ml) and purified on a Sephadex G-50 gel filtration column (2 × 75 cm), by eluting with distilled water at 0.5 ml/min. Aliquots (3 ml) were collected and pooled into fractions by monitoring absorbance at 280 nm. Fractions were lyophilised, dissolved with water (4 mg/ml), and analysed for their antioxidant properties (see 6.2.4.2). The most active fraction was lyophilised, dissolved with water (50 mg/ml), and loaded on a Sephadex G-25 gel filtration column (2 × 75 cm), by eluting, monitoring, and pooling into fractions as

described above. Similarly, the fraction with the highest antioxidant activity was further purified on a G-10 gel filtration column (2 × 75 cm), by eluting, monitoring, and pooling into fractions as described above. The most antioxidant fraction was analysed by RP-HPLC (see 6.2.4.3).

6.2.4.2 Fraction antioxidant activity

For each antioxidant assay, a Trolox aliquot was used to develop a 50-500 µmol/l standard curve. All data were then expressed as Trolox Equivalents (µmol TE/100 ml).

6.2.4.2.1 DPPH•-scavenging assay

The test was performed according to Brand-Williams, et al. (1995). Fraction aliquots (20 µl) were added to 3 ml of DPPH solution (6×10^{-5} mol/l) and the absorbance was determined at 515 nm every 5 min until the steady state.

6.2.4.2.2 Ferric Reducing Antioxidant Power (FRAP) assay

The assay was performed as previously described (Benzie and Strain 1996). A solution of 10 mmol/l TPTZ in 40 mmol/l HCl and 12 mmol/l ferric chloride was diluted in 300 mmol/l sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Fraction aliquots (20 µl) were added to 3 ml of the FRAP solution and the absorbance was determined at 593 nm every 5 min until the steady state.

6.2.4.3 Fraction purity evaluation

Sephadex G-10 fraction showing the strongest antioxidant capacity was filtered through a Phenex-PVDF 17 mm Syringe Filter 0.45 µm (Phenomenex, Torrance, CA, USA) and analysed by RP-HPLC. Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA, USA) provided with photodiode array detector (DAD). The column selected was an Aeris PEPTIDE 3.6 µm XB-C18 New Column 250 x 4.6 mm (Phenomenex). Elution conditions consisted in 2% acetic acid (solvent A) and 2% acetic acid in methanol (solvent B) gradient at a flow rate of 1.0 ml/min. The gradient conditions were: 0-20 min, 0-100% B; 20-23 min, 100% B; 23-27 min, 0% B, followed by 5 min of maintenance. Chromatograms were recorded at 280 nm and indicated that the fraction analysed was a high purity peptide (96%), that was named as MBCP and subjected to characterization.

6.2.5 Characterization of MBCP

MBCP was collected, and its purity and molecular weight were determined by ultra-performance liquid chromatography electro-spray ionization tandem mass spectrometry

(UPLC ESI-MS/MS). A Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA), coupled with a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer (UPLC-ESI Q-TOF MS) (Waters Corporation), was used to identify the purified peptide. MBCP was dissolved in mobile phase, and 5 µl of peptide solution was loaded onto an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 µm) (Waters Corporation). Elution conditions consisted in 0.5% acetic acid (eluent A) and methanol (eluent B) gradient at a flow rate of 0.3 ml/min. The gradient conditions were: 0–3 min, 5% B; 3–15 min, 5%–30% B; 15–17 min, 30% B. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectroscopy analysis. MS/MS analyses were performed in the positive electrospray ionization mode by using CID. Collision energy was selected from 10 to 35 eV. Argon was introduced as the collision gas at a pressure of 10 psi. Sequencing of peptide was acquired over the m/z range 50–2000 using the Biolynx software.

6.2.6 Peptide Synthesis

MBCP was synthesised using the solid-phase method and purified via HPLC after deprotection. Synthetic peptide was used to confirm MBCP identity. Both synthetic and isolated MBCP were used for *in vitro* assays and results were compared.

6.2.7 CaCo2 cell line tests

6.2.7.1 Cell culture and proliferation assay

The assay was performed according to previous authors (De Simone, et al. 2009). CaCo2 cells (American Type Culture Collection, Rockville, MD) were grown in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and supplemented with 12.5% foetal calf serum (FCS) (Flow, McLean, VA, USA), 1% (v/v) MEM non-essential amino acids, 5 mM L-glutamine, 1% sodium pyruvate, 40 U/ml penicillin, 100 µg/ml gentamycin, and 40 µg/ml streptomycin (DMEMc). The cells (17–21 passages) were grown in a humidified atmosphere of CO_2/air (5:95) at 37 °C and were plated in 12 multi-well plates at different densities. After incubation for 4 h, the cells were washed with PBS to remove unattached dead cells. The cells were pre-treated for 30 min with 50 µM H_2O_2 in PBS solution (H-CaCo2). Then, the pre-confluent H-CaCo2 (21 passages) cells were seeded in 12 multi-well plates and incubated at 37 °C for 24 h with 0.0125 to 1 mg/ml of MBCP in water solution. The cell number was measured with a haemocytometric counter and cell proliferation was evaluated by CyQuant® cell proliferation assay Kit (InvitrogenTM) (Thermo Fisher Scientific, San Jose, CA, USA) with dye fluorescence

measurement at 480 nm excitation maximum and 520 nm emission maximum. The control sample consisted in H-CaCo2 cells not added of peptide (untreated cells). Cell proliferation was expressed in percentage of proliferation compared with the control. All experiments were performed on triplicate cultures.

6.2.7.2 Preparation of cell extract

The assay was performed as previously described (Tenore, et al. 2014). H-CaCo2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM ethylenediaminetetraacetic acid (EDTA). The cells were sonicated, followed by centrifugation at 13000 x g for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below.

6.2.7.3 Measurement of intracellular ROS accumulation

The assay was performed as previously described (Gomez-Monterrey, et al. 2013). The thiobarbituric acid reactive substances (TBARS) assay was performed on membranes extracted from Caco-2 and H-Caco2 cells after 48 h incubation with 0.0125 to 1 mg/ml of MBCP in water solution, and from the untreated control cells, using an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors. The homogenate was centrifuged at 1200 × g for 10 min to separate the cytosol (supernatant) from the membranes (pellet). The pellet was dissolved in 50 mM Tris, 150 mM NaCl, and 10 mM EDTA. Aliquots (10 µl) of the membrane preparation were added to 2 mL thiobarbituric acid (TBA) trichloroacetic acid (TCA) (15% TCA, 0.3% TBA in 0.12 M HCl) solution at 100 °C for 30 min. The reaction was stopped by cooling the sample in cold water. The samples were centrifuged at 5000 × g for 10 min and the chromogenic (TBARs) quantified by spectrophotometry at a wavelength of 532 nm. The quantity of TBARs was expressed as µM/µg proteins.

6.2.7.4 Measurement of cellular superoxide dismutase activity

The assay was performed as previously described (Tenore et al., 2014). Total cellular superoxide dismutase (SOD) activity was measured as follows (Kirshenbaum and Singal 1992). Briefly, a reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/ml catalase, 70 µM nitro blue tetrazolium, 0.2 mM xanthine, 50 µM bathocuproinedisulphonic acid, and 0.13 mg/ml bovine serum albumin (BSA). A 0.8 ml aliquot of the reaction mixture was added to each cuvette, followed by addition of 100 µl of lysate. The cuvettes were pre-warmed at 37 °C

for 3 min. The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve and expressed as units per mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method which makes use of BSA as the standard.

6.2.7.5 In vitro intestinal transepithelial transport studies

The assay was performed as previously described (Tenore et al., 2014). The human colon carcinoma cell line CaCo2 (HTB-37) was obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were cultured (17-21 passages) in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and supplemented with 12.5% foetal calf serum (FCS), 1% nonessential amino acids, 5 mM L-glutamine, 40 U/ml penicillin, 100 µg/ml gentamycin, and 40 µg/ml streptomycin (DMEMc). Cells were maintained at 37 °C in a humidified atmosphere of CO_2/air (5:95, v/v) and passaged every 7 days by trypsinization. They were seeded in transwell (Transwell® inserts of 3 µm pore size and 24 mm diameter) at 6 x 104 cells/cm2. The medium (15 mL DMEM containing 12.5% FCS) was changed every 2 days until cells reached confluence (7-8 days). The integrity of the monolayers (cultured for 14–15 days) was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Zug, Switzerland). Only CaCo2 monolayers showing TEER higher than 300 Ω x cm^2 were used for the experiments. The integrity of the monolayers was checked before, during and after the experiment. Then, CaCo2 cells monolayers were gently rinsed twice with PBS, medium was removed from the apical and basal sides of the cultures, transport medium (TM, Hank's balanced salt solution supplemented with 25 mM glucose and 10 mM HEPES) was added to the apical (2 ml) and to the basolateral (2 ml) compartments, and pH was adjusted to 6 or 7.4. After 30 min of incubation, medium at the apical side was replaced with fresh TM containing MBCP 0, 0.5, 1, 2 or 4 mM. After 4 h of incubation at 37 °C, apical and basal solutions were collected, and aliquots (5 ml) were filtered on Phenex-PVDF 17 mm Syringe Filter 0.45 µm (Phenomenex, Torrance, CA). Samples were stored at -20 °C until LC-MS/MS analyses to measure the concentration of MBCP in both compartments (for LC-MS/MS operating conditions, see sub-section 6.2.5).

6.2.8 Human erythrocyte test

6.2.8.1 Haemolysis inhibition assay

The inhibitory capacity of MBCP on erythrocyte haemolysis was determined according to the procedures reported by Helmerhorst, et al. (1999) with slight modification. Blood was obtained from healthy volunteers. The experiment was carried out in accordance with the guidelines issued by the Ethical Committee of the Istituto Superiore di Sanità (2003). Erythrocytes were separated from plasma by centrifuging at 1200 g for 10 min at 4 °C, and washed three times with PBS. Then, erythrocyte suspension (0.2 ml, 20%, v/v, dissolved with PBS) was mixed with MBCP solution (0.2 ml, 0.1 to 2 mg/ml), and was gently shaken and incubated at 37 °C for 30 min. At the end of incubation, 0.2 ml of 100 mM H_2O_2 in PBS solution was added. The mixture was further incubated at 37 °C for 2 h. After incubation, the mixture was diluted with 3.2 ml PBS (pH 7.4). After centrifuging at 1200 g for 10 min at 4 °C, the absorbance of the supernatant was read at 540 nm. Distilled water was used as control. The inhibition of erythrocyte haemolysis was calculated as $(1-A_{sample}\ 540/A_{control}\ 540) \times 100\%$.

6.2.8.2 Effect on intracellular ROS generation

In order to clarify the anti-haemolytic mechanisms, the potential effects of MBCP against H_2O_2-induced oxidative stress in human erythrocytes was evaluated.

Erythrocyte suspension was obtained and incubated with MBCP solutions as described in sub-section 6.2.8.1. After addition of 0.2 ml of 100 mM H_2O_2 in PBS solution and incubation at 37 °C for 2 h, the mixture was centrifuged at 1200 g for 10 min at 4 °C. The residue erythrocytes were washed 3 times with PBS (pH 7.4) and re-suspended with 5 volumes of PBS (pH 7.4). An aliquot (100 µl) of erythrocytes suspension was firstly centrifuged at 1200 g for 10 min at 4 °C and the supernatant was discarded. Then, DCFH-DA (200 µl, 10 µmol/l) was added to suspense the erythrocytes. After incubation at 37 °C for 25 min in the dark, the mixture was washed with PBS to completely remove the DCFH-DA outside the erythrocytes. At the end of washing, the erythrocytes containing the fluorescent probe were re-suspended with 600 µl PBS. Intracellular ROS generation was measured by recording the fluorescence intensity of erythrocytes by a Varioskan Flash Spectral Scan Multimode Plate Reader (Thermo Fisher Scientific, Waltham, MA), with the excitation and emission wavelengths at 485 and 525 nm, respectively. PBS-treated erythrocytes were considered as the blank control with 100% of the fluorescence intensity. The results were reported as the percentage of DCF fluorescence intensity of control (% control) calculated as following: DCF Fluorescence (% control) = F_{sample} x 100%/F_{blank},

where F_{sample} and F_{blank} represent the fluorescence intensity of sample and blank control, respectively.

6.2.9 Statistics

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The level of significance (α-value) was 95% in all cases ($P < 0.05$).

6.3 Results and discussions

6.3.1 Isolation and identification of MBC peptides after gastrointestinal digestion

The gel filtration chromatography was used for the fractionation of MBC intestinal digest. Two fractions (Fractions A and B) were obtained from Sephadex G-50 column (Fig. 8A) and their antioxidant activity was evaluated. Since natural antioxidants are characterised by complex reactivity and different mechanisms of action, the antioxidant capacity of food and food extracts cannot be assessed by using a single method (Schlesier, et al. 2002). Then, two different spectrophotometric assays, DPPH and FRAP tests, were executed, and antioxidant activities were expressed as Trolox equivalents (TEs). The sample ability to scavenge free radicals is indicated by the decrease in DPPH absorption, while the FRAP test evaluates the reducing power of sample constituents.

As shown in Table 10, all the fractions showed antioxidant capacity, and Fraction B exhibited the highest values. Fraction B was further purified on a Sephadex G-25 column (Fig. 8B) to obtain three fractions (Fractions 1-3). The most active one (Fraction 3) was further fractionated on Sephadex G-10 column (Fig. 8C) to yield three fractions (Fractions I-III) (Table 10). Fraction III showed the strongest antioxidant capacity (Table 10) and was chosen to be further purified by HPLC. As shown in Fig. 8D, HPLC analysis showed four peaks of which peak at retention time (Rt) 23.4 min represented about 96% of the fraction. Thus, HPLC analysis indicated that Fraction III was a high purity peptide that was named as MBCP and subjected to characterization.

Q-TOF MS analysis indicated for MBCP a molecular mass of 1165.45 Da. The observed molecular mass was in agreement with the calculated molecular mass of the peptide. MS/MS analysis showed that the major sequence of MBCP was Cys-Lys-Tyr-Val-Cys-Thr-Cys-Lys-Met-Ser (CKYVCTCKMS), which was referred to a novel peptide, in

accordance to databases available online (http://www.prospector.ucsf.edu; http://www.expasy.ch).

Pure MBCP was used as external standard for HPLC-DAD quantitative analysis. A stock solution was prepared in water and dilutions were made to cover a concentration range of 0.012–0.36 mg/ml. MBCP was quantified as 180.89 mg/100 g MBC fresh weight (fw).

In order to ascertain the presence of MBCP in the non-digested MBC, an MBC aliquot (250 g) was subjected to preparation, purification, and fraction characterization, as reported above. The absence of such peptide in the native MBC corroborated the hypothesis that MBCP was the enzymatic product of MBC simulated GI digestion.

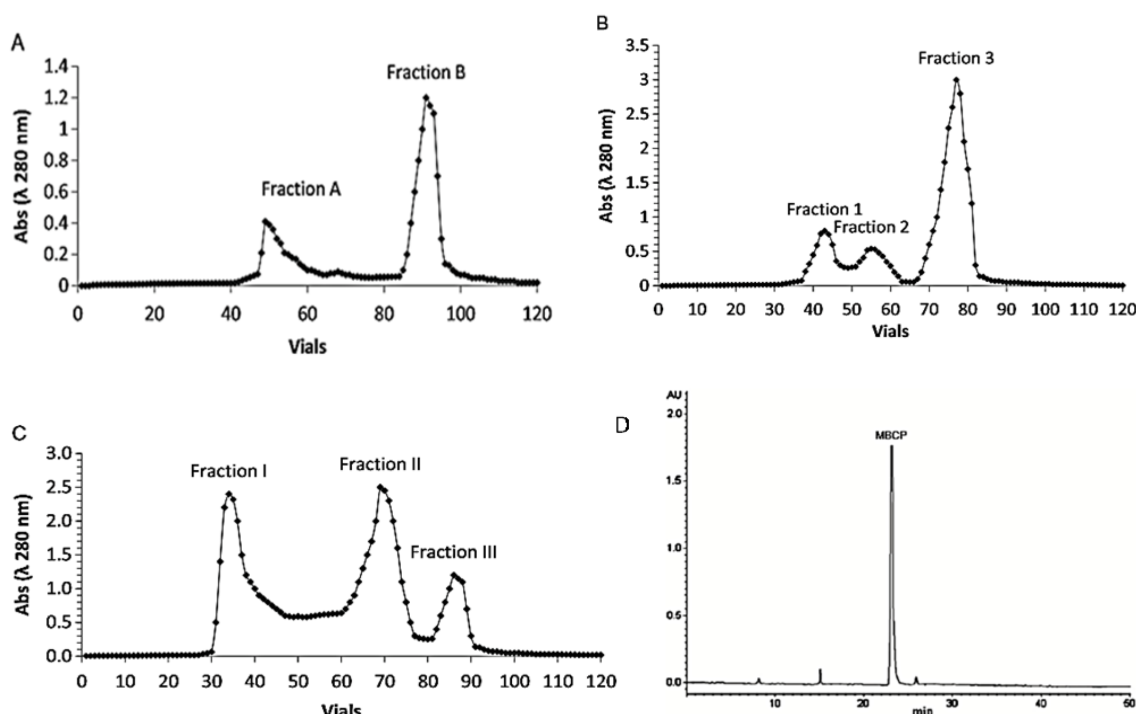

Fig. 8 *Chromatographic isolation of MBCP.*
(A) Fractionation of MBC gastrointestinal digest by Sephadex G-50 gel filtration chromatography; (B) fractionation of fraction B by Sephadex G-25 gel filtration chromatography; (C) fractionation of fraction 3 by Sephadex G-10 gel filtration chromatography; (D) RP-HPLC analysis of fraction III.
Abbreviations: MBC: "Mozzarella di Bufala Campana DOP"; MBCP: "Mozzarella di Bufala Campana DOP" peptide.

Table 10. *Near equilibrium steady state antioxidant capacity of size exclusion chromatographic fractions from MBC intestinal digest **

	Assay method	
Fractions	DPPH	FRAP
Sephadex G-50*		
A	137.53 ± 2.10^{a}	98.25 ± 1.86^{a}
B	161.39 ± 2.61^{b}	125.77 ± 1.72^{b}
Sephadex G-25**		
1	157.53 ± 1.93^{c}	120.18 ± 2.76^{c}
2	143.09 ± 3.11^{d}	112.43 ± 2.16^{d}
3	163.98 ± 2.72^{e}	126.37 ± 2.58^{e}

Sephadex G-10***		
I	139.09 ± 3.21^f	102.43 ± 2.35^f
II	129.64 ± 2.83^g	86.59 ± 1.96^g
III	164.84 ± 3.25^h	114.76 ± 1.93^h

*Expressed as TEs (μmol/l) of fractions measured by FRAP and DPPH assays at the steady state (DPPH, 45 min; FRAP, 55 min). Data are mean values ± SD ($n = 5$; $P < 0.05$).

[*] The concentration of all fractions was 4 mg/ml.

[**] The concentration of all fractions was 0.1 mg/ml.

[***] The concentration of all fractions was 0.05 mg/ml.

[abcdefgh] Mean values in columns with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test ($P < 0.05$).

6.3.2 MBCP effects on H_2O_2-stressed CaCo2 cell lines

CaCo2 cell lines derived from human intestinal epithelial adenocarcinoma are regarded as a suitable model system for the *in vitro* evaluation of intestinal functions and nutrient absorption. Particularly, they are reported to reproduce several of the normal physiological responses to various modulatory agents, in a model which mimics the damages deriving from exposition to endogenous and exogenous oxidative agents (Levy, et al. 1995).

Thus, the potential protective effects of MBCP against H_2O_2-induced oxidative stress in CaCo2 cell lines was evaluated. Cells were exposed to 50 μM H_2O_2 for 30 min (H-CaCo2) and then treated for 24 h with 0.0125 to 1 mg/ml of MBCP. A linear correlation between incubation dose and cell proliferation was revealed, and the maximum result was achieved with a 0.2 mg/ml MBCP dose which made the stressed cell line proliferation increase by about 100% (Fig. 9A). I examined the effect on free radical and manganese superoxide dismutase levels in the stressed cell line exposed to increasing doses (0.0125-1 mg/ml) of MBCP (Fig. 9B). Our data demonstrated that MBCP at a maximum dose of 0.2 mg/ml was able to directly scavenge free radicals without interfering with cell antioxidant defensive system involving enzymes and proteins for self-protection.

The structure–function relationship and the mechanism of peptide-induced antioxidant activity have not been fully elucidated. The mechanism of action has been referred to the physicochemical properties of the peptide quali-quantitative amino acid profile. As regards our peptide composition, amino acids, such as Tyr, Met, Lys, and Cys, are generally accepted to be antioxidants (Wang and De Mejia 2005). Particularly, residues with an aromatic ring structure (such as Tyr) can donate a proton to electron deficient radicals, while non-polar residues (Val and Met) can enhance the solubility of peptides in a lipid matrix improving the accessibility to hydrophobic radical species or polyunsaturated fatty acids (Power, et al. 2013). Further structural properties, which are important predictors of a peptide antioxidant activity, refer to the polar domain of the C terminal amino acid, and the

low hydrophobicity, together with steric hindrance, of the second amino acid adjacent to the C terminus. These aspects make amino acids, such as Ser and Met, respectively, frequently present in these positions (Power et al., 2013). Nevertheless, the sulphydryl (R-SH) group in Cys has a unique antioxidant activity and interacts with the radical species by hydrogen donation from the SH group (Elias, et al. 2008).

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

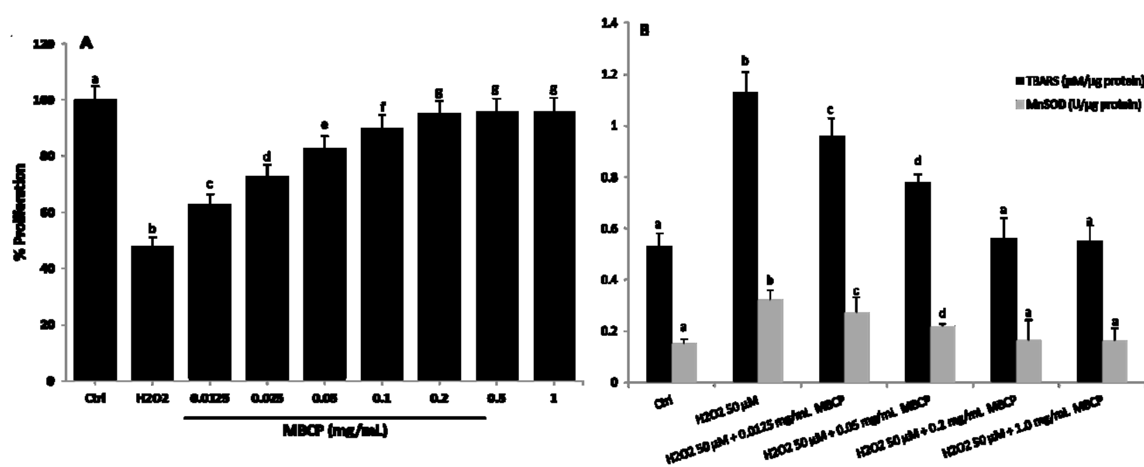

Fig. 9 – *Effects of MBCP on H_2O_2-stressed CaCo2 cell lines.*
(A) Enhancement of cell proliferation; (B) protection against H_2O_2-induced ROS generation.
MBCP: “Mozzarella di Bufala Campana DOP” peptide. Ctrl: untreated cell lines. TBARS: thiobarbituric acid reactive substances; MnSOD: manganese superoxide dismutase.
Values are expressed as means ± SD (n = 5; $P < 0.05$).
[abcd] Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test.

6.3.3 In vitro MBCP intestinal stability and bioavailability

The intestinal stability and bioavailability of MBCP were evaluated by using single layers of CaCo2 cells as a model of absorption in the small intestine.

LC-MS analysis highlighted no significant hydrolysis (less than 10%) of MBCP in the apical solution by the brush border exopeptidases after 120 min incubation and regardless of the peptide concentration (Fig. 10A). LC-MS analysis of basolateral solution revealed that MBCP was absorbed intact through CaCo2 monolayer, with a concentration-dependent transport following a saturable pattern described by a linear curve (Fig. 10B). For LC-MS operating conditions, see sub-section 6.2.5. Interestingly, the actual amount of MBCP transepithelially transported was about 10%, thus, higher than what generally reported for different size (3 to 17 amino acid units) and polarity peptides transported from CaCo2 monolayer apical to basolateral side (Pauletti, et al. 1997; Regazzo, et al. 2010;

Satake, et al. 2002). Among the many different transport pathways, the carrier-mediated transport systems, such as the H^+-coupled PepT1 transporter, may be excluded as main pathway involved in the transport of MBCP since they are active and saturable symporters specific for intestinal absorption of charged di- and tripeptides (Brandsch, et al. 2008). The average polar properties of MBCP would also exclude a possible passive transcellular diffusion since a vesicular-mediated internalization, the main mechanism involved, would imply absorption by apical cell membrane through hydrophobic interactions (Knipp, et al. 1997). Actually, the low level of degradation of MBCP during its transepithelial transfer strongly corroborates that passive transcellular diffusion would not be the main pathway involved in its transport. Nevertheless, the capacity of amino acids to form hydrogen bonds with lipid phosphates of cell membranes, like that of most of MBCP amino acid moieties (Cys, Lys, Tyr, Thr, Ser), has been described as a main physicochemical feature favouring the translocation process via transcytosis route (Pauletti, et al. 1996), so that the passive diffusion would not be completely excluded. Overall, a possible involvement of the paracellular route in the transport of MBCP could be hypothesised. In fact, the passive paracellular transport via tight junctions has been usually reported to be normally applicable to the absorption of water soluble low-molecular-weight and short-chain peptides and, in general, it is specific for positively charged molecules because tight junctions are on average negatively charged (Salamat-Miller and Johnston 2005).

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

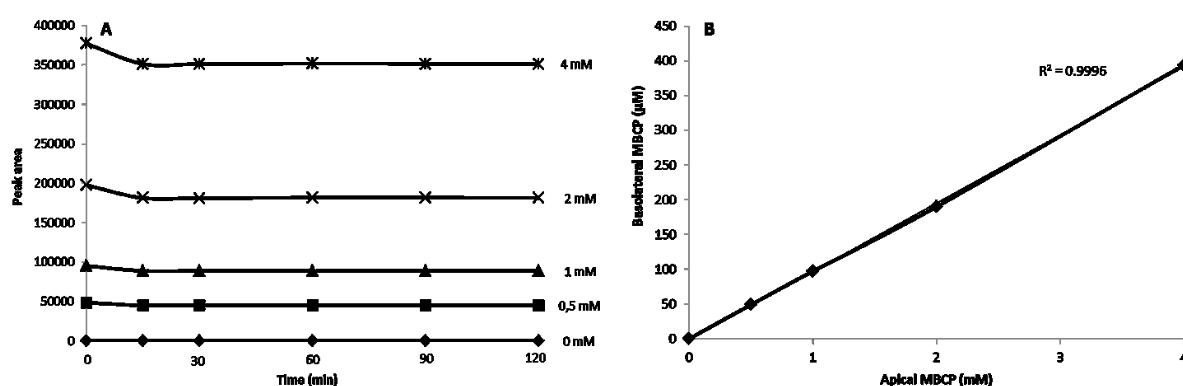

Fig. 10 *In vitro MBCP intestinal stability and bioavailability.*
(A) Stability of MBCP to the brush border exopeptidases measured as change in LC–MS chromatogram peak area of MBCP introduced at different concentrations in the apical compartment of CaCo2 cell monolayer.
(B) Quantification of MBCP in CaCo2 cell monolayer apical and basolateral solutions using a five-point calibration curve of pure MBCP as standard analysed by LC–MS.
Abbreviations: MBCP: “Mozzarella di Bufala Campana DOP” peptide.

6.3.4 *MBCP effects on H_2O_2-induced human erythrocyte haemolysis*

Since a significant amount (about 10%) of MBCP was shown to be transported from CaCo2 monolayer apical to basolateral side, MBCP would be expected to be potentially able to reach blood circulation and exert systemic effects. Primary targets could be red blood cells; interestingly, recent studies have reported on both erythrocyte protecting and haemolytic capacities of food deriving peptides (Ghribi, et al. 2015; Hong, et al. 2015; Xue, et al. 2009).

MBCP was observed to possess a strong ability to inhibit H_2O_2-induced human erythrocyte haemolysis (Fig. 11A). The inhibition ratio was 41.38% at 0.1 mg/ml of MBCP, and reached 91.25% when the concentration increased to 2.0 mg/ml. The estimated half-inhibitory concentration (IC50) was 0.12 ± 0.01 mg/ml.

In order to clarify the anti-haemolytic mechanisms, the potential effects of MBCP against H_2O_2-induced oxidative stress in human erythrocytes was evaluated. The nonpolar DCFH-DA was used as a probe to investigate the generation of ROS. DCFH-DA can rapidly diffuse into cells and is hydrolysed by cellular esterases into DCFH. The intracellular DCFH can be easily oxidized by free radicals into fluorescent DCH. The fluorescence intensity increases with the increase of intracellular ROS (Wolfe and Liu 2007). Our data clearly indicated for MBCP a significant capacity to counteract H_2O_2-induced oxidative stress in red blood cells (Fig. 11B). Particularly, ROS generation was reduced by 43.28% at 0.1 mg/ml of MBCP and reached 92.18% when the concentration increased to 2.0 mg/ml.

All of the reported results were in full agreement with those obtained by performing the same assays on the synthetic MBCP.

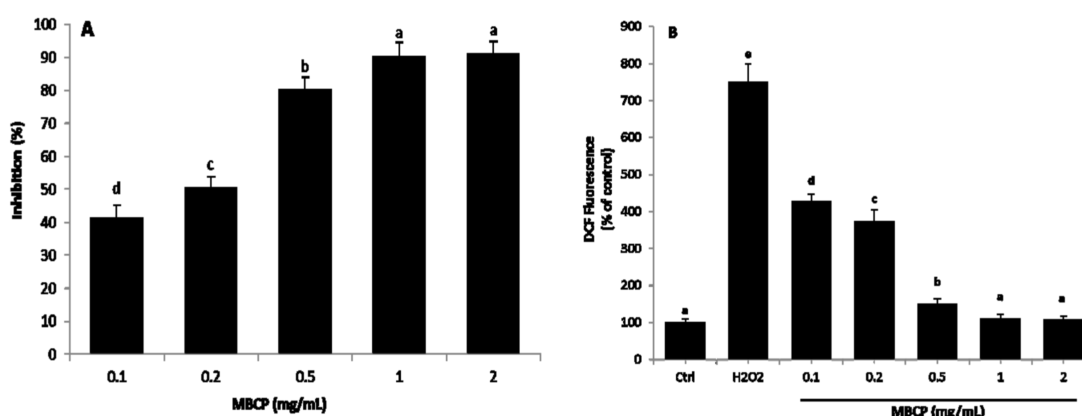

Fig.11 *Protective effect of MBCP against H_2O_2-induced human erythrocyte haemolysis*.
(A) Inhibition of erythrocyte haemolysis; (B) Effect on ROS generation in erythrocytes.

Abbreviations: MBCP: “Mozzarella di Bufala Campana DOP” peptide; Ctrl: untreated cell lines.
Values are expressed as means ± SD (n = 5; $P < 0.05$).
[abcd] Mean values with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test.

6.4 Conclusions

The present study indicated MBC as a good source of bioactive peptides after GI digestion. Specifically, a novel antioxidant peptide (MBCP) was detected in the intestinal digest and its *in vitro* intestinal protection, bioavailability, and anti-haemolytic capacity were assayed. A potential intestinal protection against induced oxidative stress was revealed. MBCP demonstrated a good stability to brush border exopeptidases and a surprisingly higher bioavailability than what generally reported by *in vitro* experiments for different size and polarity peptides. Concerning the significant amount of MBCP transepithelially transported, MBCP would be expected to be potentially able to reach blood circulation and exert systemic effects. According to recent studies reporting on erythrocyte protecting capacities of food deriving peptides, MBCP was observed to possess a strong ability to inhibit H_2O_2-induced human erythrocyte haemolysis. Our experimental results would suggest MBC as a potential functional food and MBCP as a novel food ingredient, food additive and pharmaceutical, relevant in health promotion and disease risk reduction.

7 Bioactive peptide derived from gastrointestinal digestion of "Mozzarella di Bufala Campana PDO": *in vitro* and *in vivo* intestinal anti-inflammatory effect and a clinical study.

7.1 Introduction

Since the intestine represents a critical defence barrier against luminal toxic agents, an alteration of intestinal mucosa, mainly known as *leaky gut syndrome* or high intestinal permeability (Michielan and D'Inca 2015), is the basis of intestinal inflammatory diseases.

Inflammatory bowel disease (IBD) is the main disease involved in high intestinal permeability, characterised by chronic inflammation of the gastrointestinal tract. Crohn's disease and ulcerative colitis are the principal events of IBD. Although the aetiology of IBD is still unknown, it arises as a result of the interaction of environmental or genetic factors and the immune responses (Sairenji, et al. 2017).

IBD treatment involves the use of anti-inflammatory drugs (such as 5-aminosalicylic acid), corticosteroids, monoclonal antibodies [anti-TNF-α antibodies] and vascular adhesion molecules (anti-integrin antibodies) (Lucafò, et al. 2018; Sairenji, et al. 2017). However, these drugs are not curative, and they are mainly used as induction and maintenance therapy. Moreover, for most of these drugs, a high inter-individual variability in a positive response has been reported (Lucafò, et al. 2018). Therefore, it is essential to find clinical efficacious alternatives for the treatment of IBD.

Recently, intestinal permeability has been recognised as a new target for IBD prevention and therapy (Fasano and Shea-Donohue 2005; Michielan and D'Inca 2015). The architecture and function of the intestinal epithelium requires close coordination between enterocyte proliferation and apoptosis, both highly dependent on cell–cell and cell–matrix interactions. Enterocytes are joined to each other by tight junctions and adherens junctions. The main molecular component of the adherens junctions is E-cadherin, a transmembrane protein with five extracellular domains that interdigitate with those of an adjacent cell in a calcium-dependent homophilic manner to form a continuous linear "zipper" structure (Brüser and Bogdan 2017; Coopman and Djiane 2016). E-cadherin exerts functional adhesion activity when it is connected to the actin cytoskeleton by the cytoplasmic complex α-, β-, or γ-catenin in a mutually exclusive manner (Brüser and Bogdan 2017; Coopman and Djiane 2016). It is well established that, in IBD patients, these junctional complexes undergo a disruption leading to a breaking of the intestinal barrier (Edelblum and Turner 2009). Moreover, mucosal biopsies from patients with IBD have increased

levels of cytokines, able to regulate intercellular permeability, such as interferon-gamma (IFN-γ) and TNF-α (Utech, et al. 2006).

As reported in chapter six, a novel strong peptide identified from buffalo cheese (MBCP) after simulated *in vitro* gastrointestinal digestion, is able to reduce H_2O_2-induced oxidative stress in intestinal epithelial cells and in erythrocytes, as well as MBCP has good stability to brush border exopeptidases and a high bioavailability. Thus, the second aim was to evaluate the therapeutic potential of MBCP in IBD, investigating the effect of MBCP on (i) adjacent junctions *in vitro* permeability under inflammatory conditions in an intestinal epithelial cell line, (ii) intestinal inflammation and the associated changes in motility in mice, and (iii) the effect of a novel nutraceutical formulation based on MBCP composition in a clinical study.

7.2 Material and methods

7.2.1 Chemicals

All chemicals and reagents used were of analytical grade. 2,4,6-dinitrobenzenesulfonic acid (DNBS), croton oil, fluorescein isothiocyanate (FITC)-conjugated dextran (molecular mass 3–5 kDa), betamethasone, atropine, tubocurarine and neutral red (NR) solution were purchased from Merck Life Science (Milan, Italy). TNF-α was obtained from R&D Systems, Space Import-Export SRL (Milano, Italy). All reagents for cell cultures were obtained from Sigma, Bio-Rad Laboratories (Milan, Italy) and Microtech Srl (Naples, Italy).

7.2.2 Cell culture

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA), a human colorectal adenocarcinoma epithelial cell line, were grown at 37 °C in a 5% CO_2 atmosphere in h-glucose Minimum essential medium MEM containing 1% non-essential amino acids and supplemented with 10% de-complemented fetal bovine serum (FBS), 100 $U·ml^{-1}$ penicillin, 100 $mg·ml^{-1}$ streptomycin, 1% l-glutamine and 1% sodium pyruvate. The growth of the cells was measured by counting the cells with a Coulter counter (Nexcelom, Lawrence, MA, USA, Cellometer Auto1000). All experiments were performed in triplicate.

7.2.3 Animals

Male adult ICR mice, weighing 20–25 g for upper gastrointestinal transit experiments and 25–30 g for colitis experiments, were purchased from Charles River Laboratories (Calco,

Lecco, Italy) and housed in polycarbonate cages under controlled temperature (23 ± 2 °C), constant humidity (60%) and a 12 h light/dark cycle. The animals were acclimatised to their environment at least 1 week under the above reported standard conditions with free access to tap water. Mice were fed ad libitum with a standard rodent diet, except for the 24 h immediately before the intracolonic administration of DNBS or the oral administration of the charcoal meal and for 2-h before the oral gavage of croton oil or MBCP. Mice were randomly allocated to different experimental groups and outcome assessments were performed in single-blind. All experiments were approved by the Institutional Animal Ethics Committee for the use of experimental animals and conformed to guidelines for the safe use and care of experimental animals in accordance with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council (86/609/ECC and 2010/63/UE).

7.2.4 Cytotoxicity Studies

We evaluated the effect of MBCP on Caco-2 cell viability by using a microplate colorimetric assay that measures the ability of viable cells to incorporate and bind the neutral red (NR), a weak cationic dye, in lysosomes. Cells were plated at the appropriate density to obtain a model of undifferentiated and differentiated cells; then, 5 × 103 and 20 × 103 cells per well in 96-well plates for undifferentiated and differentiated cells, respectively. After 24 hours, cells were exposed to various concentrations of MBCP (9–375 μM) for 48-h. Then, cells were incubated with NR dye solution (50 μg/ml) for 3 h at 37 °C, and finally washed with phosphate buffered saline (PBS) and lysed with 1% acetic acid. The absorbance was read at 540 nm (iMarkTM microplate reader, Bio-Rad, Milano, Italy).

7.2.5 Immunostaining and Confocal Microscopy

After 24 h and 48 h of incubation with TNF-α (10 μM), TNF-α plus MBCP (18 μM) or TNF-α plus betamethasone (10 μM), Caco-2 cells were fixed for 20 min with a 3% (w/v) paraformaldehyde solution and permeabilized for 10 min with 0.1% (w/v) Triton X-100 in PBS at room temperature. To prevent nonspecific interactions of antibodies, cells were treated for 2 h in 5% BSA in PBS. In another set of experiments, Caco-2 cells were treated with the acetylcholine receptor antagonists atropine (10 μM) and tubocurarine (10 μM) for 6-h. Immunostaining was carried out by incubation with anti-E-cadherin, anti-actin and anti-β-catenin antibodies (1:500, Alexa Fluor®, BD PharmingenTM). The slides were mounted on microscope slides by Mowiol. The analyses were performed with a Zeiss LSM

510 microscope equipped with a plan-apochromat objective X 63 (NA 1.4) in oil immersion. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

7.2.6 Permeability Assay on Caco-2 Cells

To evaluate cell permeability, Caco-2 cells were seeded into a transwell filters with a pore diameter of 8 μm in 24-well plates at a density of 2.0 × 105 cells/insert. Further cultivation in the same medium as above allowed the cells to spontaneously differentiate and polarize into the epithelial monolayer within 21 days. The basolateral compartment contained 1.5 mL of culture medium while apical compartment contained 0.2 ml. After seeding, the cells were treated with 0.07 µM of MBCP. The culture medium was replaced 3 times/week. After 21 days, the filters containing the cell monolayers (with or without MBCP) were treated for 2 h with TNF-α (10 µM) and then with a mannitol-lactulose solution (0.05 mmol/l, 0.25 mol/l, 0.2 ml). After 3 hours, the basolateral solution was collected and to determine the lactulose/mannitol ratio (LMR) through by liquid chromatography-mass spectrometry (LC-MS) analysis.

In another set of experiment, Caco-2 cells were seeded with MBCP peptide at the final concentration of 0.005 mg/ml, 100 µl of 0.25 mM lactulose and 100 µl of 0.05 mM mannitol. After 2 h of incubation at 37 °C apical and basal solutions were collected to determine the LMR through LC-MS analysis. The negative control was prepared without the addition of the aforementioned peptide.

7.2.6.1 Evaluation of LMR by LC-MS analysis

Analyses were performed on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) coupled to an Advion Expression mass spectrometer (Advion Inc., Ithaca, NY) equipped with an Electrospray (ESI) source. Mass spectra were recorded in negative SIM mode. The capillary voltage was set at − 150 V, the spray voltage was at 3 kV, the source voltage offset was at – 25 V and the capillary temperature was set at 300 °C. According to Kubica, et al. (2012), the chromatographic separation was tested on analytical column Luna-NH_2 (150 × 4.6mm, id. 3mm, 100 Å) and security guard column both supplied by Phenomenex (Torrance, CA, USA). The analyses were performed at flow rate of 1 ml/min, with solvent A (ACN + 0.05% HCOOH) and solvent B (0.05% HCOOH). Elution was performed according to the following linear gradient: from 75% (A) to 40% (A) in 6 min. The injection volume was 10 µl and the column temperature was fixed at 30 °C. Each sample was injected in triplicate. For quantitative analyses stock solutions of lactulose and mannitol were prepared by dissolving them in a mixture 75:25 acetonitrile/water (Kubica,

et al. 2012). Standard curves were prepared over a concentration range of 0.1-1.0 µg/µl with six different concentration levels and triplicate injections at each level.

7.2.7 Induction of Experimental Colitis

Colitis was induced in the anesthetized mice by the intracolonic administration of 2,4,6-dinitrobenzene sulfonic acid (DNBS) as previously described (Pagano, et al. 2016). Briefly, DNBS (150 mg/kg), dissolved in 50% ethanol (150 µl/mouse), was administrated into the distal colon using a polyethylene catheter (1 mm in diameter) via the rectum (4.5 cm from the anus). Three days after the DNBS administration, all mice were euthanised by asphyxiation with CO_2, the mice abdomen was opened by a midline incision and the colon removed, isolated from surrounding tissues, length measured, opened along the antimesenteric border, rinsed and weighed and then fixed in 10% formaldehyde for histopathological analysis. MBCP was administrated by oral gavage (10–100 mg/kg) once a day for 3 consecutive days starting 24 h after DNBS administration. Animals were euthanised 2 hours after the last administration of MBCP.

7.2.8 Haematoxylin-Eosin Staining

Paraffin-embedded colon tissues were cut into 5-mm sections. The sections were dewaxed in xylene for 10 min and dehydrated in gradient alcohol. The sections were then stained with haematoxylin for 8 min and eosin for 5 min. After dehydration, the sections were sealed and examined under a light microscope (Leica DM 2500). Photographs were taken using the Leica DFC320 R2 digital camera (Venditti, et al. 2018).

7.2.9 Immuno-Fluorescence Microscopy

The fixed slides of colon tissue, were dewaxed, rehydrated and processed. Briefly, antigen retrieval was performed by pressure-cooking slides for 3 min in 0.01 M citrate buffer (pH 6.0). To prevent nonspecific interactions of antibodies, the slides were treated for 2 h in 5% BSA in PBS. Immunostaining was carried out by incubation, overnight at 4 °C, with anti-E-cadherin and anti-β-Catenin antibodies (1:100, Alexa Fluor®, BD PharmingenTM). The slides were mounted on microscope slides by Mowiol + DAPI for nuclear staining, and then observed under the optical microscope (Leica DM 5000 B + CTR 5000).

7.2.10 Intestinal Permeability Measurement

The effect of MBCP, at a dose of 100 mg/kg, was also tested on intestinal permeability, using the FITC-Dextran method. Briefly, 2 days after the induction of colitis, mice were gavage with 600 mg/kg of FITC–conjugated dextran (molecular mass 3–5 kDa). After 24

h, blood was collected by cardiac puncture, and the FITC-derived fluorescence was immediately analysed in the serum by a microplate reader (GloMax Explorer System, Promega; excitation wavelengths 485 ± 14 nm, emission wavelengths 520 ± 25 nm). Serial-diluted FITC dextran was used to generate a standard curve. Intestinal permeability was expressed as the concentrations of FITC (μM) detected in the serum.

7.2.11 Induction of Intestinal Hypermotility and Upper Gastrointestinal Transit in Mice

Upper gastrointestinal transit was measured in both physiological and pathological conditions. The hypermotility was induced by the inflammatory agent croton oil (CO) as previously described (Capasso, et al. 2014). Briefly, two doses of CO (20 μl/mouse) were given for two consecutive days by oral gavage and the upper gastrointestinal transit was measured 4 days after the first administration of CO (Pol and Puig 1997) (i.e., when the maximal inflammatory response associated to the intestinal hypermotility was reported). Mice were deprived of food overnight and then the upper gastrointestinal transit was evaluated by identifying the leading front of an intragastrical administered charcoal meal marker (10% charcoal suspension in 5% gum Arabic, 10 ml/kg) in the small intestine as previously described (Capasso, et al. 2014). Twenty minutes after charcoal administration, mice were euthanised, and the small intestine was isolated by cutting at the pyloric and ileocecal junctions. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum. MBCP was administrated by oral gavage (5–50 mg/kg) 30 min prior to charcoal administration.

7.2.12 Study population

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in July 2018. Patients aged 25-69 years were eligible for enrolment if they showed intestinal inflammatory diseases, such as inflammatory bowel diseases (Crohn's disease, Ulcerative colitis), irritable bowel syndrome, or diseases with damage to the villi (celiac disease, sprue, giardiasis). Exclusion criteria were as follows: smoking, obesity (BMI > 30 kg / m^2), diabetes, hepatic disease, renal disease, heart disease, family history of chronic diseases, intense physical exercise (> 10 h/week), pregnant women, women suspected of being pregnant, women who hope to become pregnant, breastfeeding, birch pollen allergy and blood donation less than 3 months before the study. The subjects received oral and written information regarding the study before they gave their written consent. Protocol, letter of intent of volunteers and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento,

Italy). The study was approved by the committee and carried out in accordance with the Helsinki Declaration of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a randomized, double-blind, monocentric, placebo-controlled trial. The study duration was 16 weeks: the group underwent 4 weeks of run-in, followed by 8 weeks of nutraceutical treatment, and 4 weeks of follow-up. Both the examinations and the study treatment were performed in an outpatient setting. Clinic visits and urine sampling were performed at weeks 0, 4, 8, 12 and 16. Clinic visits, urine sampling and blood sampling to test hepatic and renal toxicity, were performed after 12 h of fasting at weeks 0, 4, 8, 12 and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h before blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10-ml EDTA-coated tubes (Becton-Dickinson, Plymouth, United Kingdom) and plasma was isolated by centrifugation (20 min, 2200 g, 4C). Urine samples were collected in urine sterilised box. All samples were stored at -80° C until analysis. Plasma aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (c-GTP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, total bilirubin, and creatinine levels were determined on a Diacron International Free Carpe Diem, using commercially available kits from Diacron International (Grosseto, Italy). Urine analysis of LMR were determined on HPLC-MS system (see section 7.2.6.1).
In addition to these five meetings, five standardised telephone interviews were performed starting from the first meeting to verify compliance and increase protocol adherence. In particular, these interviews reminded patients to complete their intake checking table for the intervention study and to record any discontinuation or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit). All patients underwent a standardised physical examination, assessment of medical history (for up to 5 years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. At each clinic visit, patients had to complete three self-administered questionnaires on quality-of-life aspects, and their diaries were checked for data completeness and quality of documentation to ensure patient comprehension of the diary items.

7.2.13 Study treatment

The group of 40 patients (24 men and 16 women) was randomly divided into two subgroups (each one of 20 subjects). All subjects assuming capsules (500 mg each one)

were instructed to take two capsules at lunch, and two capsules at dinner. Treatment group take capsules of lyophilized amino acids mix (Cys-Lys-Tyr-Val-Thr-Met-Ser in ratio 3:2:1:1:1:1:1), while placebo group took identically appearing capsules containing only maltodextrins, for 8 weeks. Both groups took 50 mL of an aqueous solution at 66.7% w/V of lactulose and 18% w/V of mannitol at the beginning of the study (T_0), after 30 days (T_{30}) and after 60 days (T_{60}). The sugars solution was composed by 10g of lactulose and 5 g of mannitol. A urine sample was collected after 5 h from the ingestion of sugars solution. At the end of the administration period, a 4-week follow-up period was carried out.

7.2.14 Randomization, concealment and blinding

A total of 40 eligible patients (24 men and 16 women) were randomly assigned to two groups to receive the treatment or placebo. Supplements and placebo were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. If a patient dropped out before receiving supplement, he or she was replaced by the next eligible patient enrolled at the same centre. The concealed allocation was performed by an Internet-based randomization schedule, stratified by study site. The random number list was generated by an investigator without clinical involvement in the trials. Patients, physicians, lab technicians and trial staff (data analysts, statisticians) were blind to treatment allocation.

7.2.15 Study outcomes and data collection

7.2.15.1 Primary and secondary efficacy outcomes.

The primary endpoint measured was intestinal permeability variations, while key secondary outcomes collected during clinic visits were measurements of blood pressure and heart rate. All raw patient ratings were evaluated in a blinded manner at the site of the principal investigator. The decision process was performed according to a consensus document (unpublished standard operating procedure) before unblinding to define conclusive primary and secondary efficacy data from a clinical perspective.

7.2.15.2 Safety

We assessed safety from reports of adverse events as well as laboratory parameters concerning hepatic and renal function, vital signs (blood pressure, pulse, height and weight), and physical or neurological examinations. Safety was assessed over the entire treatment period at weeks 0, 4, 8, 12 and 16 including adverse events occurring in the first 3 weeks after cessation of treatments.

7.2.16 Statistic

7.2.16.1 Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) (Little and Rubin 2019), but also provides reasonably stable results even when the assumption of MAR is violated (Molenberghs, et al. 2004; O'Kelly and Ratitch 2014). Patients who did not provide any diary data (leading to zero evaluable days) were excluded from the MAR based primary efficacy analysis, according to an "all observed data approach" as proposed by White, et al. (2012). This approach is statistically efficient without using multiple imputation techniques (Carpenter and Kenward 2007). Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed by using Cochran's test ($p<0.1$). The I^2 statistic was also calculated, and $I^2>50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percent change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, p-values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration, total polyphenols dose, and Jadad score. Treatment effects were analysed using PROC MIXED with treatment and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum parameters and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P < 0.05$).

7.2.16.2 Analysis sets

The full analysis set population included all randomized patients and patients who did not fail to satisfy a major entry criterion. We excluded patients who provided neither primary nor secondary efficacy data from efficacy analyses. The per protocol set consisted of all patients who did not substantially deviate from the protocol; they had two characteristics. First, this group included patients for whom no major protocol violations were detected (e.g., poor compliance, errors in treatment assignment). Second, they had to have been on treatment for at least 50 days counting from day of first intake (completion of a certain prespecified minimal exposure to the treatment regimen). Hence, patients who prematurely discontinued the study or treatment were excluded from the per protocol sample.

7.2.17 Patient involvement

No patients were involved in setting the research question or the outcome measures, nor were they involved in developing plans for participant recruitment or the design and implementation of the study. There are no plans to explicitly involve patients in dissemination. Final results will be sent to all participating sites.

7.2.18 Statistical Analysis

Data are expressed as the mean ± S.E.M. or S.D. of n experiments. Statistical significance was assessed using the Student's t-test for comparing a single treatment mean with a control mean, and a one-way ANOVA followed by a Tukey multiple comparisons test for the analysis of multiple treatment means. Values of $p < 0.05$ were considered significant. The IC_{50} (concentration that produced 50% inhibition of cell viability) values were calculated using sigmoidal dose response curve-fitting models (Graphpad Prism Software, version 5.03, Inc. Avendia de la Playa La Jolla, CA, USA).

7.3 Results

7.3.1 In vitro study

7.3.1.1 MBCP Induces Cytotoxic Effects in Caco-2 Cells at Very High Concentrations

In order to characterise a safety profile of MBCP, we evaluated its effect on the cell viability of pre- (undifferentiated exponentially growing) and post-confluent (differentiated) Caco-2 cells. MBCP (9–375 μM), induced in undifferentiated and differentiated Caco-2 cells, a concentration-dependent cell viability inhibition at 48 h with an IC_{50} of 65 μM (Figure 12). Therefore, to characterize the anti-inflammatory effects of MBCP on Caco-2 cells, subsequent experiments were performed using a MBCP

concentration that did not induce significant cytotoxic effect in both pre and post-confluent cells (i.e., 18 μM).

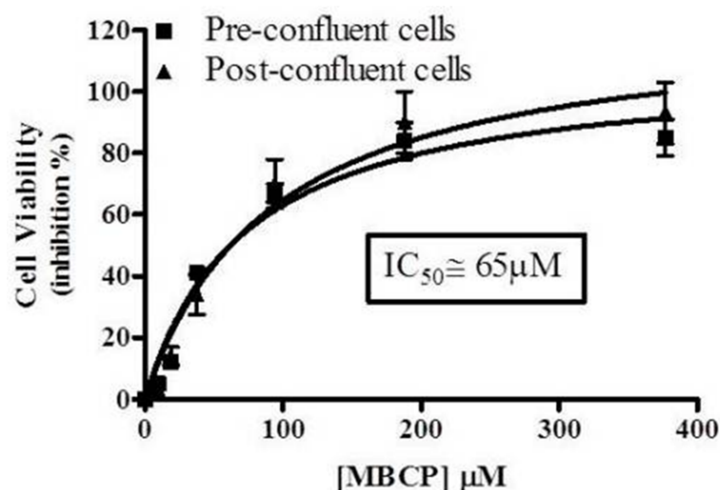

Fig. 12 *MBCP reduces cell viability in Caco-2 cells.*
Cell viability was evaluated by the Neutral Red assay in pre and post-confluent Caco-2 cells. Cells were incubated with increasing concentration of MBCP (9–375 μM) for 48 h. Each point represents the mean ± SD of three independent experiments.

7.3.1.2 MBCP Modulates Adherens Junctions Formation in Control and TNF-α-Stimulated Caco-2 Cells

In order to investigate the role of MBCP on adherens junctions (AJs) formation, we studied the cellular organization of E-cadherin and actin in pre-confluent Caco-2 cells by confocal microscopy. Caco-2 cells, after 24 h from seeding, showed a strong cytoplasmic localization of both E-cadherin and actin proteins (Figure 13A–C) that was ameliorated by MBCP treatment (Figure 13D–F). At 48 h, the E-cadherin-actin complex presented a not homogeneous membranous localization in untreated Caco-2 cells (Figure 13G–I). MBCP treatment induced a strong cell–cell contact membranous localization of E-cadherin-Actin complex (Figure 13M–O). To assess whether MBCP could counteract the AJ-destructuration induced by the pro-inflammatory mediator TNF-α, we examined the β-catenin organization in TNF-α-stimulated cells. Caco-2 cells treated with TNF-α (10 μM, for 48-h) showed a cytoplasmic compartment β-catenin localization (Figure 14D–F) compared to untreated cells (CTR) (Figure 14A–C). A treatment of Caco-2 cell with MBCP (18 μM) induced an enhancement of both E-cadherin and β-catenin localization at AJs (Figure 13G–I) compared to TNF-α alone. Since corticosteroids are the effective first-line treatment in cytokine-induced inflammation, we treated the Caco-2 cells (stimulated with TNF-α) with betamethasone. Betamethasone (10 μM) treatment induced a

membranous E-cadherin and β-catenin organization, although a β-catenin cytoplasmic immunoreactivity persisted (Figure 14L–N). In our experimental conditions (Caco-2 cells treated or not with TNF-α), MBCP accelerated and induced an organization of adjacent junctions in the cells, without changes the E-cadherin, actin and β-catenin protein expression evaluated by Western blot analysis (data not shown).

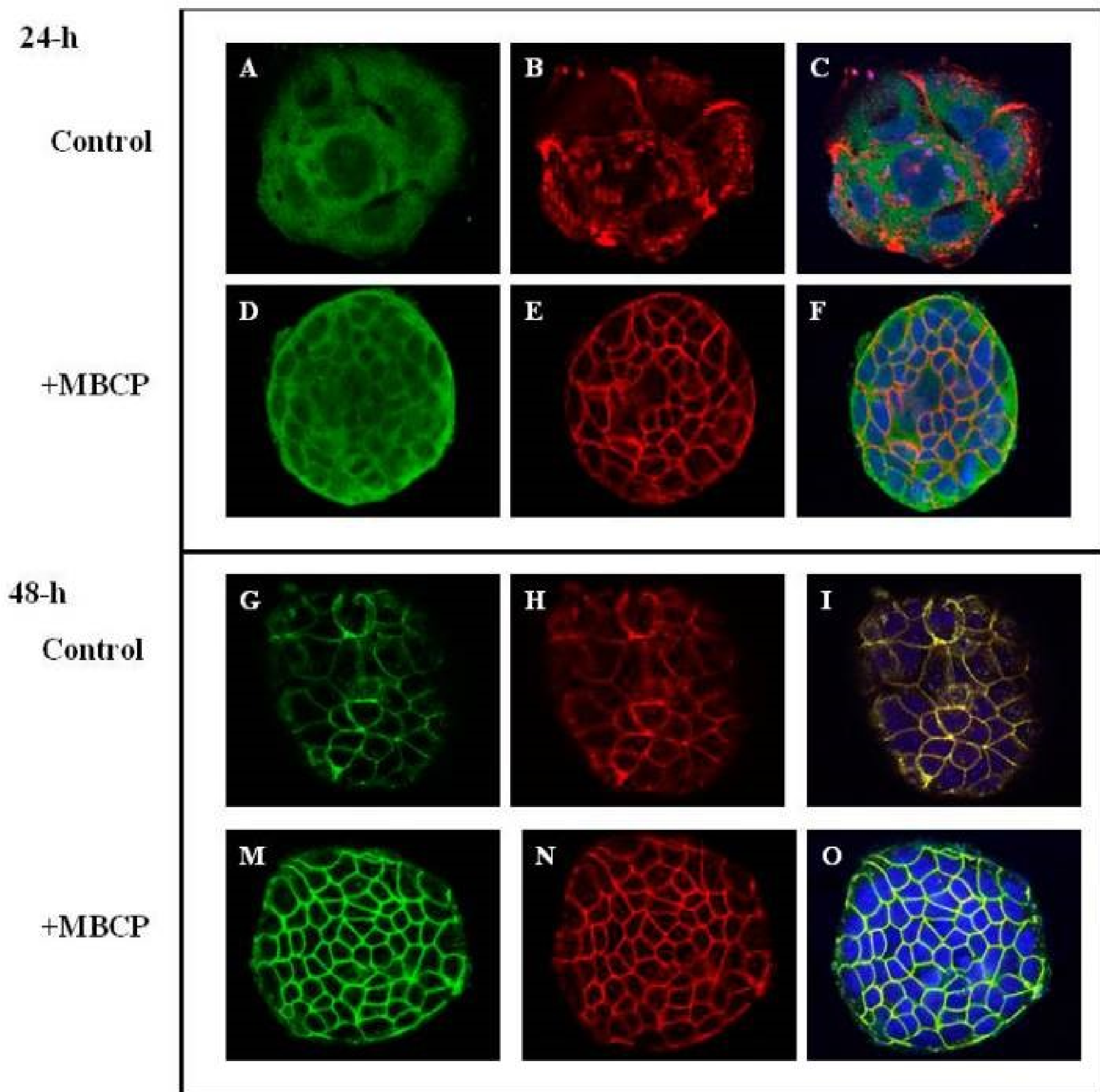

Fig. 13 *MBCP accelerates the formation of adherens junctions*.
Confocal microscopy images of Caco-2 cells (A–C, G–I) and MBCP treated Caco-2 cells (panel (D–F, M–O). Cells were treated for 24-h and 48-h with MBCP at 18 μM concentration. The merged image is on the right (green, actin; red, E-cadherin; blue, dapi).

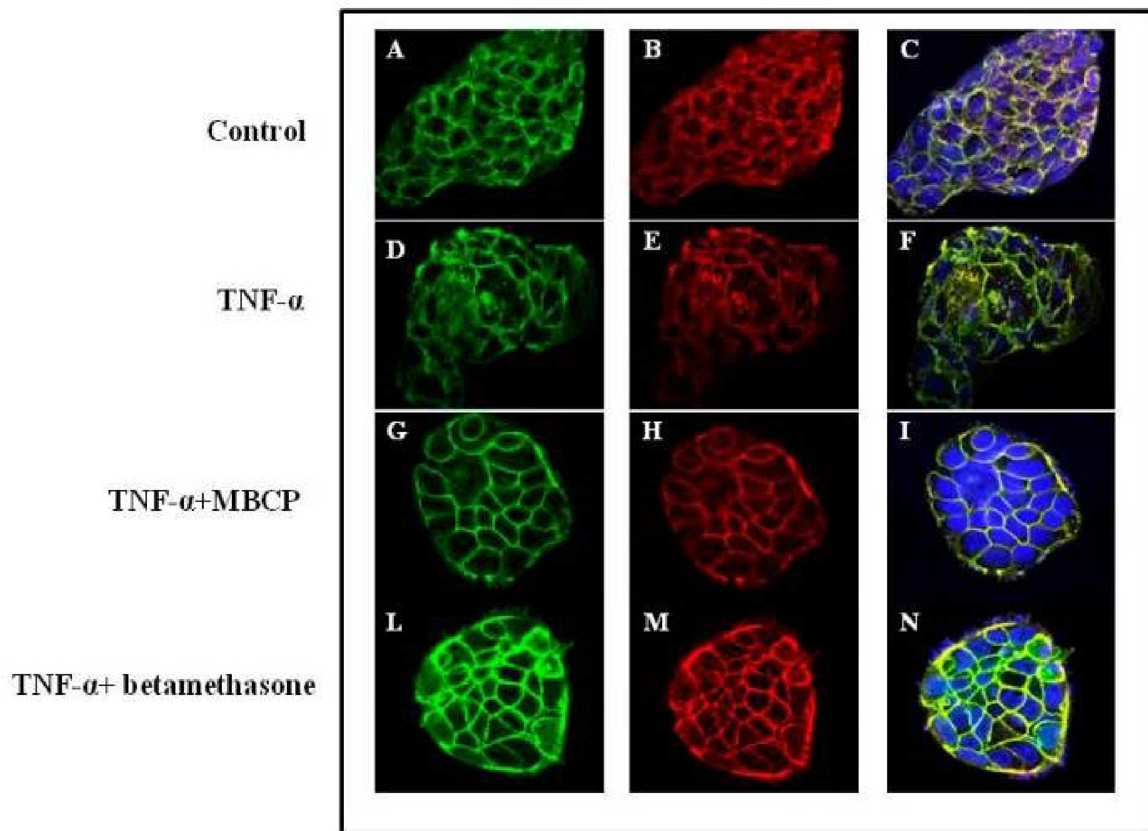

Fig. 14 *MBCP ameliorates the adherens junctions destructured by TNF-α.*
Confocal microscopy images of untreated Caco-2 cells (panel (A–C), Caco-2 cells treated for 48 h with TNF-α (10 µM, (D–F)), TNFα 10µM plus MBCP (18 µM, (G–I)) or with TNF-α (10 µM) plus betamethasone (L–N). On the right, merged image (green, β-catenin; red, E-cadherin; blue, dapi).

7.3.1.3 MBCP Counteracts the TNF-α Inflammatory Effect by Modulating the NF-κB Pathway

It is well known that TNF-α causes the activation of transcription factors, including NF-κB. NF-κB regulates host inflammatory, immune responses and also stimulates the expression of inducible cyclooxygenase enzymes (COX-2) that contribute to the pathogenesis of the inflammatory process. Therefore, we evaluated, by Western blot analysis, the effects of MBCP on NF-κB, pNF-κB, COX-2 and 5-Lipoxygenases (LOX) expression in TNF-α treated Caco-2 cells. A treatment with TNF-α induced an increase of 5-LOX, p-NF-κB and COX-2 expression. MBCP (18 µM) was able to reduce the expression of p-NF-κB, 5-LOX and COX-2 expression increased by TNF-α (Figure 15). Moreover, MBCP (18 µM) reduced the basal expression of NF-κB in untreated Caco-2 cells. Betamethasone (10 µM), used as a positive control, significantly reduced 5-LOX expression, but not the NF-κB, p-NF-κB and COX-2 expression, increased by TNF-α.

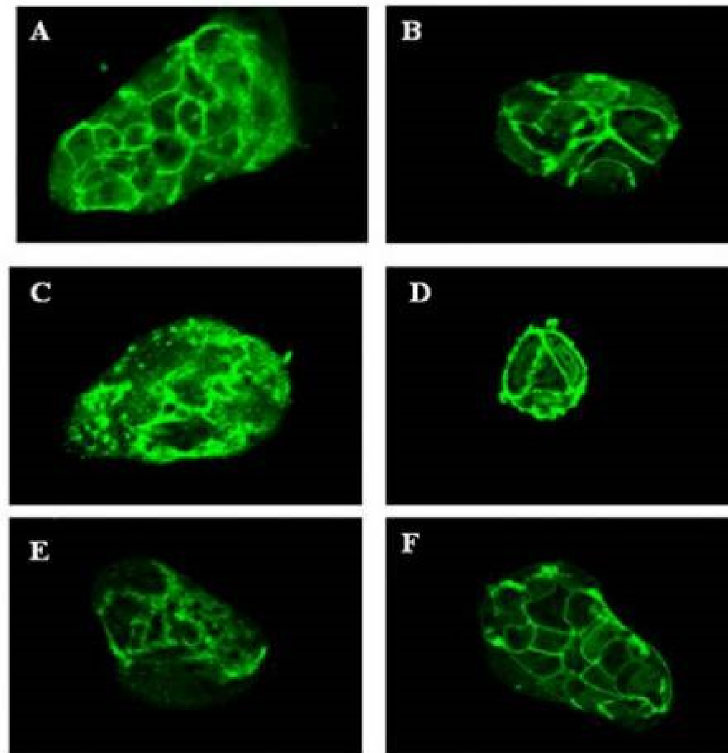

Fig. 15 *MBCP ameliorates the adherens junctions destructured by atropine and tubocurarine*.
Confocal microscopy images of untreated Caco-2 cells (A) or Caco-2 cells treated for six hours with MBCP (18 μM, (B)), tubocurarine (10 μM, (C)), Tubocurarine plus MBCP (D), atropine (10 μM, (E)) and atropine plus MBCP (F). The colour green represents β-catenin.

7.3.1.4 MBCP Inhibits the TNF-α-Increased Caco-2 Permeability

The intestinal epithelial cell line Caco-2 has been used extensively as a model of the human epithelium, as it can be grown in the Transwell system as a differentiated cell monolayer that has selective paracellular permeability to ions and solutes. We used both mannitol and lactulose, intestinal permeability probes, to evaluate the permeability changes in Caco-2 stimulated with TNF-α. Treatment of the Caco-2 cell (see Section 7.2.6) with TNF-α (10 μM) increased the concentration of mannitol in the basolateral solution by about twofold compared to the control cells (cells without TNF-α treatment). [Control of mannitol concentration (mmol/l): 0.01 ± 0.004; TNF-α 0.023 ± 0.005 *; mean ± SD; * $p < 0.05$]. The mannitol concentration in the basolateral side of the Caco-2 cells pre-treated with MBCP and then incubated with TNF-α (0.009 ± 0.003 mmol/l, mean ± SD; n = 3, * $p < 0.05$) was decreased twofold compared to the TNF-α treated Caco-2 cells. The MBCP induced any change of the lactulose permeability in the same inflammatory cell condition.

7.3.1.5 MBCP ameliorates Caco-2 intestinal permeability

Analysing graphically (Figure 16) the LMR in different stages of Caco-2 cell lines incubated with MBCP peptide, it is possible to notice how LMR is lower than the control

culture, expect for ninth day of differentiation (1.75). In the thirteenth (1.8) and twenty-first day of incubation (0.03), instead, the reduction of the sample cultures LMR, compared to control, is confirmed.

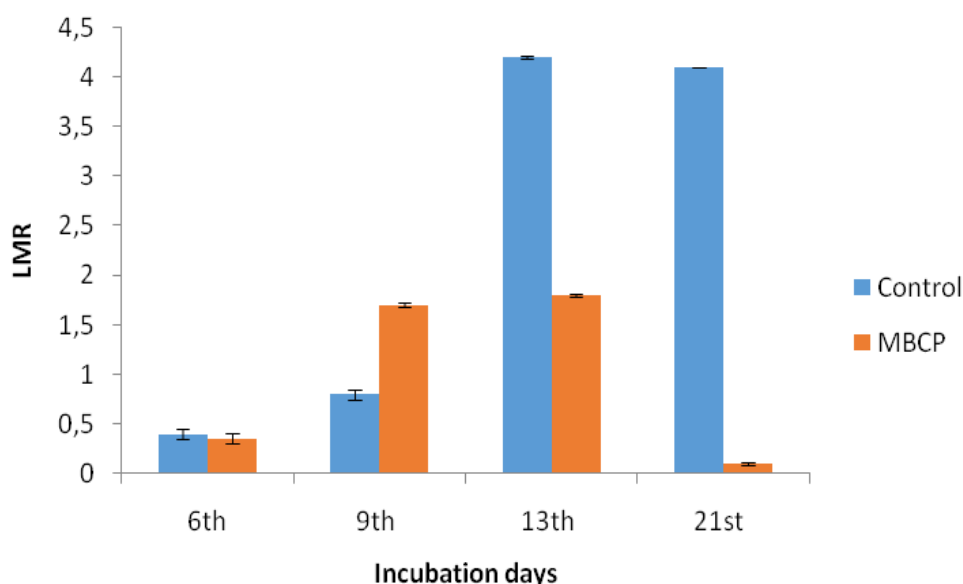

Fig. 16 *Evaluating of lactulose/mannitol ratio on Caco-2 cell lines during incubation days*
Abbreviations: LMR, lactulose/mannitol ratio

7.3.2 *In vivo study*

7.3.2.1 MBCP Reduces the Inflammation in the DNBS Model of Colitis

The DNBS murine model of colitis was used to assess the *in vivo* intestinal anti-inflammatory effects of MBCP. DNBS administration (150 mg/kg) caused inflammatory damage, as indicated by the approximately twofold increase in colon weight/colon length ratio (mg/cm), a simple and reliable marker of inflammation and damage (Figure 17A). MBCP (10–100 mg/kg, by oral gavage), administered for three consecutive days after the inflammatory insult, significantly and in a dose-dependent manner, reduced the effect of DNBS on colon weight/colon length ratio. The effect was significant starting from 30 mg/kg dose (Figure 17A). The anti-inflammatory effect MBCP was further confirmed by histological analysis. As shown in Figure 17B, DNBS caused a severe inflammatory cellular infiltration and complete destruction of the colon epithelium, compared to control mice (Figure 17B). Our data showed that oral MBCP (100 mg/kg) reduced the colonic damage induced by DNBS (Figure 17B).

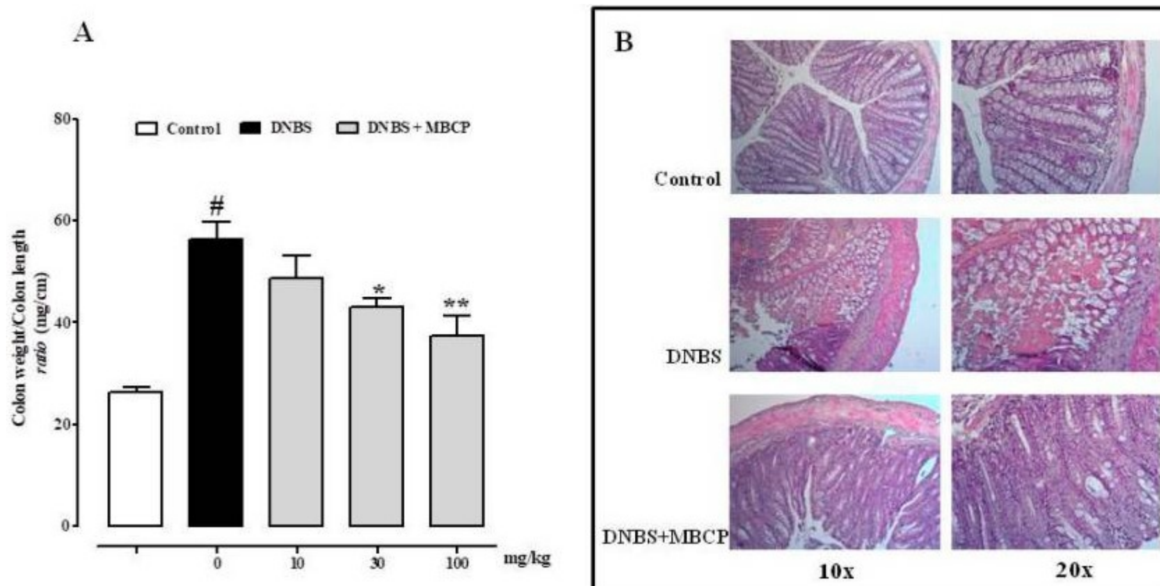

Fig. 17 *MBCP reduces colon weight/colon length ratio in DNBS-induced colitis in mice*.
(A) MBCP (10–100 mg/kg, by oral gavage) was administered once a day starting 24 h after the induction of colitis by DNBS (150 mg/kg). Colons were collected three days after DNBS. (B) representative haematoxylin and eosin stained colon cross-sections of mice treated with vehicle (control), DNBS and DNBS plus MBCP (100 mg/kg by oral gavage). Colons were collected three days after the induction of colitis by DNBS. Original magnification 10× and 20×.
(A). All data are represented as mean ± SEM of seven mice for each experimental group. Statistical significance was calculated using one-way ANOVA test. # $p < 0.001$ vs. control, * $p < 0.05$ and ** $p < 0.01$ vs. DNBS alone.

7.3.2.2 MBCP Reduces the Intestinal Permeability in vivo

The intestinal permeability was increased of the intracolonic administration of DNBS, as revealed by the high concentration of FITC-conjugated dextran in the serum (Fig 18). While MBCP (100 mg/kg), given by oral gavage for three consecutive days, synergistically ($p < 0.01$) partially counteracted the DNBS-induced increase in intestinal permeability (Fig. 18). Moreover, immunofluorescence analysis showed that DNBS administration caused a destructuration of the colonic AJs associated with an increase of the cytoplasmic expression of E-cadherin and β-catenin. MBCP (100 mg/kg) counteracted the effect of DNBS on AJs, (Figure 19), thus confirming the *in vitro* results on TNF-α-stimulated Caco-2 cells.

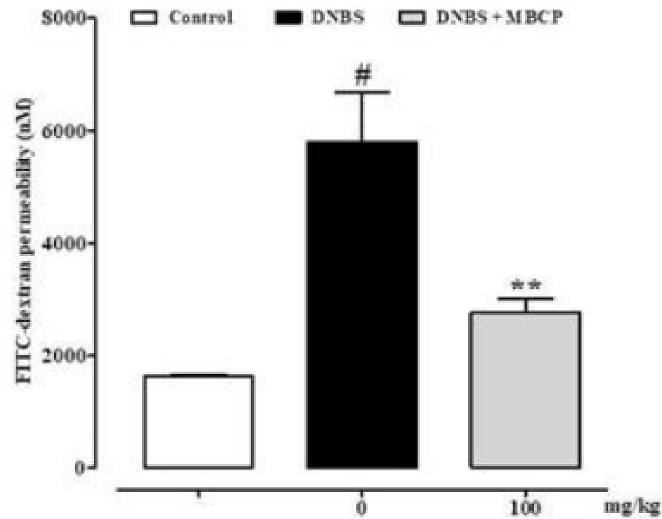

Fig. 18 *MBCP reduces intestinal permeability in DNBS-induced colitis in mice*.
Effect of MBCP (100 mg/kg, by oral gavage) on serum FITC–dextran concentration, a measure of intestinal permeability. FITC (600 mg/kg) was detected in the serum 24h after its administration and three days after the induction of colitis by DNBS (150 mg/kg).
All data are represented as mean ± SEM of 6 mice for each experimental group. Statistical significance was calculated using one-way ANOVA test. #p<0.001 vs control, **p<0.01 vs DNBS alone

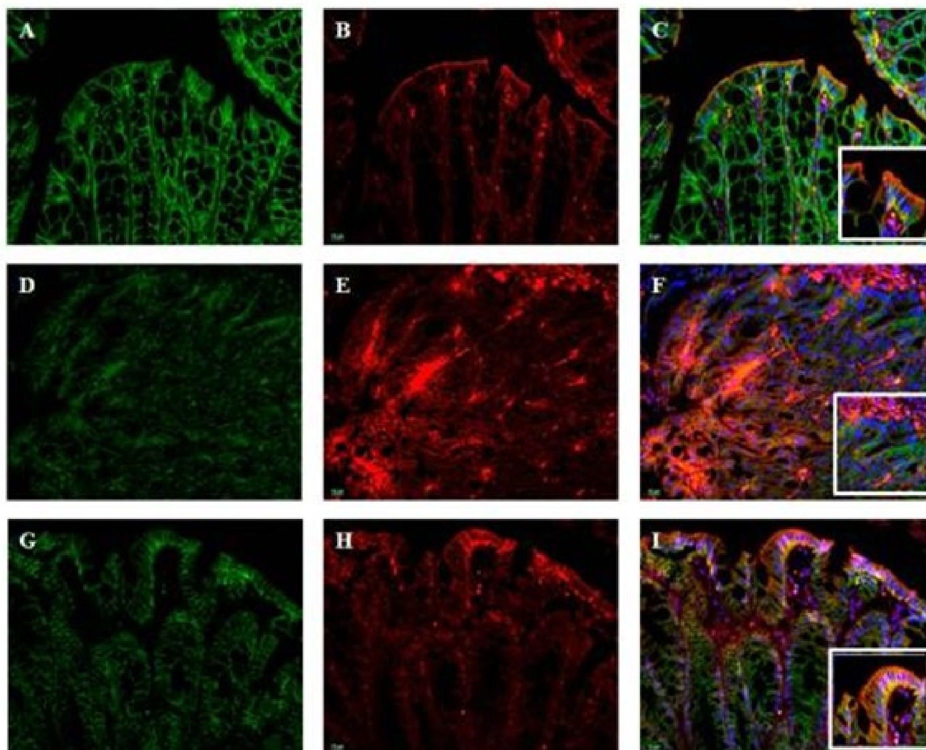

Fig. 19 MBCP *induces the organization of colonic AJs in DNBS-induced colitis in mice.* Immunofluorescence analysis showing the expression of E-cadherin (red) and β-catenin (green) in control (A–C), DNBS (D–F) and DNBS plus MBCP (G–I) (100 mg/kg) mice. Original magnification 20×. A magnified portion of the immunofluorescence was shown at higher magnification in the inset of panel (C, F, I). The DNBS-induced colitis in mice was treated for three consecutive days after the inflammatory insults with MBCP (100 mg/kg, by oral gavage).

7.3.2.3 MBCP Counteracts the Accelerated Upper Gastrointestinal Transit Induced by Croton Oil

The administration of the flogogen agent croton oil (CO) induced an accelerated upper gastrointestinal transit (GT) 4 days after its first administration. The physiological GT percentage in the mice (control) was of 49.8 ± 1.4; whereas GT significantly increased up to values of 65.50 ± 6.26* (mean ± SEM; * $p < 0.05$) in CO treated mice. MBCP (5–50 mg/kg), given by oral gavage 30 min before the administration of the charcoal, in a dose dependent manner restored the intestinal motility to physiological conditions (% of GT MBCP 5 mg/kg: 59.43 ± 2.75; MBCP 10 mg/kg: 56.14 ± 3.63; MBCP 50 mg/kg: 39.87 ± 6.38 **; ** $p < 0.01$). MBCP, at the high dose (50 mg/kg), did not modify the upper gastrointestinal transit in control mice (% of GT: control 55 ± 3.53; MBCP 48.60 ± 4.38 (mean ± SEM)).

7.3.3 Clinical study

7.3.3.1 Enrolment and subject attrition

Patients were enrolled in July 2018. A total of 56 patients were screened for eligibility; 16 patients (28.5%) did not pass the screening stage; 40 patients were randomised. The most common reason was that patients did not meet the inclusion criteria regarding inflammatory intestinal parameters (n=4), followed by concerns about the protocol (n=3), and other reasons (n=4). Some fulfilled exclusion criteria (n=5). Overall, 20 patients were assigned to the group of intervention study: they underwent a run-in period during the first 4 weeks before the treatment period of 8 weeks. Follow-up period last other 4 weeks. Figure 20 shows the flow of participants through the trials together with the completeness of diary information over the entire treatment period. No patient prematurely terminated study participation before allocation to treatment. Fig. 20 follows the CONSORT PRO reporting guideline (Raederstorff, et al. 2015) and reveals that within the assessment period, the following percentage of patients for each group provided data for the primary endpoint: treatment group 94.4% (17 of 18 patients); placebo group 84.2% (16 of 19 patients). In each group, a few patients did not submit any diaries, giving no specific reason for this. Completeness of the patient diaries did not differ between the treatment groups.

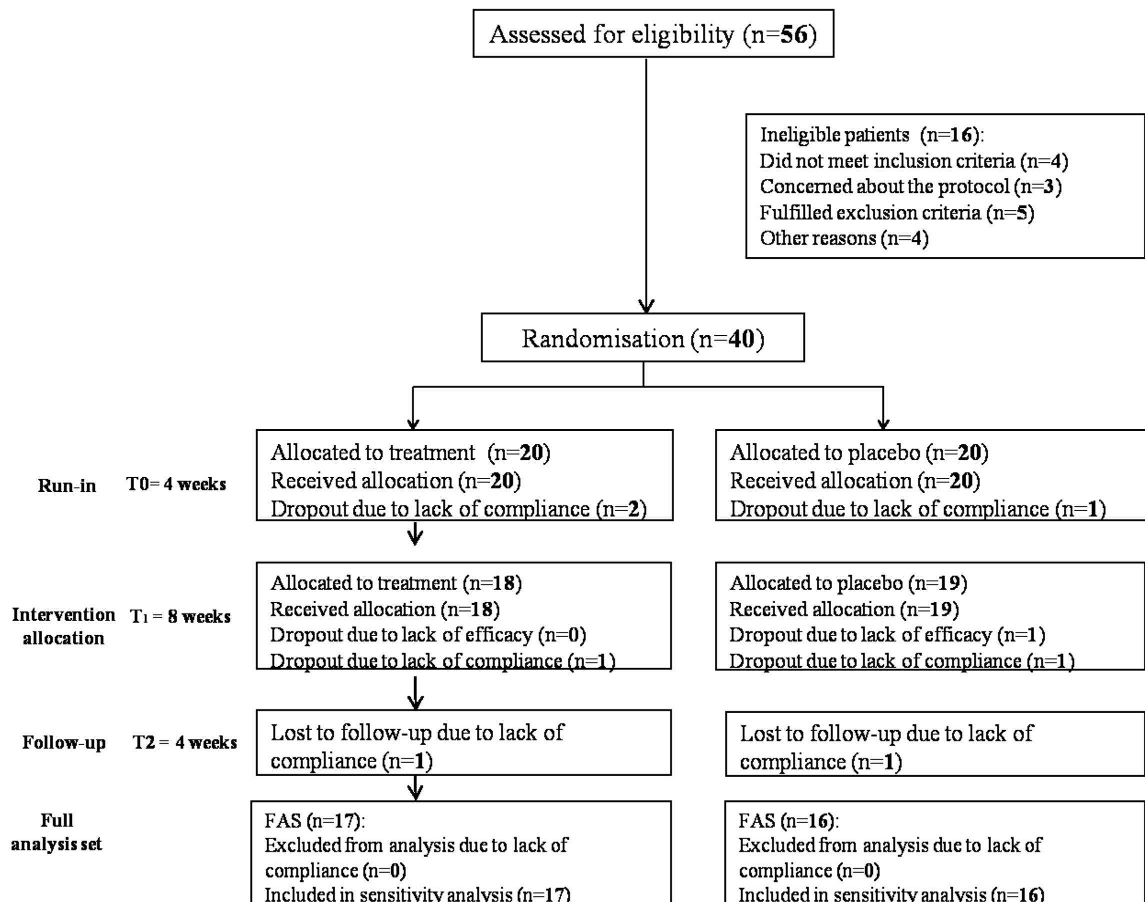

Fig. 20 *Study flowchart, according to the consolidated standards of reporting trials (CONSORT).*
The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection, and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period. FAS=full analysis set.

7.3.3.2 Participants' baseline characteristics

Table 11 shows the demographic and clinical characteristics assessed at the baseline visit of all 40 patients randomised. Overall, about half of the randomised patients were female; the total age range was 25-69 years. The groups were well balanced for demographics and clinical factors.

Table 11 *Baseline characteristics of intention to treat sample according to study treatment.*

Placebo	
Characteristics	
Demographics	
Age (years)	51.2 ± 9.8
Male sex (No %))	11 (57.9%)
White ethnicity (No (%))	19 (100%)
Clinical parameters	
LMR	1.17 ± 0.5
Treatment	

Characteristics	
Demographics	
Age (years)	50.3 ± 10.3
Male sex (No %))	9 (50%)
White ethnicity (No (%))	18 (100%)
Clinical parameters	
LMR	1.21 ± 0.6

Values are means ± SD (n=5).
Results were significantly different at a level of *P*=001.

7.3.3.3 Primary efficacy outcome measures

No significant variations of LMR, with respect to the baseline values, were registered in subjects at the end of the placebo period (Table 12). Analysing results regarding the variation of LMR values at the end of the intervention period, we can observe a significant achievement as regards LMR levels. Specifically, it was observed an average 75.2% reduction of LMR from T_0 (beginning of the study) to T_{60} (after 60th day of treatment).

Table 12 *Effects of amino acids formulations on intestinal permeability*

		Placebo	Δ%	Treatment	Δ%
LMR	T_0	1.17 ± 0.7		1.17 ± 0.94	
	T_{30}	1.19 ± 0.55	+1.7	0.58 ± 0.67 *	-50.4
	T_{60}	1.17 ± 0.79	-	0.29 ± 0.33 *	-75.2

Values are means ± SD (n=5).
T_0: 1st day of treatment; T_{30}: 30th day of treatment; T_{60}: 60th day of treatment.
* Significantly different from baseline at $P < 0.01$ (PROC MIXED).

7.3.3.4 Safety issues. study strength and limitations

All the laboratory analyses concerning the hepatic and renal function indicated no alteration of values after 2 months of lyophilised amino acids mix (Table 13). Other safety assessments, such as vital signs, blood pressure and heart rate were all periodically monitored, and baseline values did not change substantially during and at the end of the trial.

The major strengths of the clinical trial herein presented reside in the originality of the study and in the evaluation of the treatment effects in real-world settings. Conversely, the main limitations of our study include the short-term assessment for the treatment of a chronic condition, a limited cohort of enrolled patient, the choice of exclusively white race,

and the wide age range due to the availability of such individuals at the stage of the recruitment.

Table 13 Effect of amino acids mix formulation on plasma indicators of hepatic and renal function

		Treatment	Δ%
AST (GOT) (U/L)	T_0	21.6 ± 7.2	
	T_{30}	21.5 ± 5.5	-0.5
	T_{60}	21.5 ± 6.9	-0.5
ALT (GPT) (U/L)	T_0	27.9 ± 5.6	
	T_{30}	27.5 ± 4.9	-1.4
	T_{60}	27.6 ± 4.2	-1.1
γ-GTP (U/L)	T_0	37.2 ± 3.9	
	T_{30}	36.2 ± 6.3	-2.7
	T_{60}	34.4 ± 5.5	-7.5
ALP (U/L)	T_0	222.6 ± 3.1	
	T_{30}	220.4 ± 2.5	-0.98
	T_{60}	215.9 ± 3.6	-3
LDH (U/L)	T_0	177.0 ± 1.2	
	T_{30}	176.5 ± 2.6	-0.28
	T_{60}	170.2 ± 2.8	-3.84
Albumin (g/dL)	T_0	4.31 ± 0.6	
	T_{30}	4.32 ± 0.2	-0.23
	T_{60}	4.16 ± 0.5	-3.48
Total bilirubin (mg/dL)	T_0	0.57 ± 0.11	
	T_{30}	0.57 ± 0.12	-
	T_{60}	0.54 ± 0.1	-5.26
Creatinine (mg/dL)	T_0	0.84 ± 0.09	
	T_{30}	0.82 ±0.1	-2.38
	T_{60}	0.80 ±0.08	-4.76

Values are mean ± SD (n =5).
AST, aspartate aminotransferase; GOT, glutamic oxaloacetic transaminase; ALT, alanine transaminase; GPT, glutamic pyruvic transaminase; c-GTP, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase

7.4 Discussions

In mammals, the enterocytes are renewed continuously every 4–8 days through an organised series of events involving proliferation, differentiation and programmed cell

death. The proliferation to differentiation transition (PDT) is a critical step in the continual renewal of a normal intestinal epithelium (Yang, et al. 2013). A useful *in vitro* model to study the effects of food ingredients on the gut epithelial layer is based on the use of Caco-2 cells for their functional similarity to colonic enterocytes (Tanoue, et al. 2008). Indeed, Caco-2 cells express tight junctions, microvilli, enzymes and transporters functionally similar to colonic enterocyte (Tanoue, et al. 2008). Moreover, this cell line has the ability to elicit a pro-inflammatory reaction in response to stimulants like TNF-α, a known mediator of gastrointestinal mucosal barrier injury (Michielan and D'Inca 2015; Tatiya-aphiradee, et al. 2018). In this study, we have shown that a peptide (MBCP) obtained from gastrointestinal digestion of Mozzarella of Bufala Campana DOP is able to modulate the differentiation and permeability in Caco-2 cells stimulated with TNF-α and to attenuate inflammation and hypermotility in murine models of intestinal inflammation.

7.4.1 MBPC Modulates the Differentiation and Permeability in Caco-2 Cells

Proliferating Caco-2 cells spontaneously initiate the differentiation process when they have reached confluence. The differentiation program starts when specific biochemical events induce the cell–cell contact, through the E-Cadherin/actin/β-Catenin complex (Buhrke, et al. 2011). MBCP treatment, already after 24 h, induced the differentiation program accelerating the E-cadherin-actin membranous organization, thus suggesting a beneficial effect on the AJ. This result is further supported by the experiments with TNF-α. According to previous studies (Yi, et al. 2009), we have shown that Caco-2 treatment with TNF-α induced molecular alterations of the E-cadherin organization and consequently a passage of mannitol through the Caco-2 monolayer. MBCP treatment was able to restore the cell–cell junctions, counteracting the breakdown of E-cadherin-β-catenin complex and reducing the increase of mannitol permeability inducted by TNF-α.

Moreover, MBCP treatment on Caco-2 cell lines in order to evaluate the LMR, an index of human intestinal permeability, showed a result significantly lower than the control culture, expect for ninth day of differentiation. This could be justified by the fact that the cells are not yet completely differentiated, and we may expect an irregular trend.

Indeed, different results are obtained on the thirteenth day of culture, where the reduction of LMR is observed. This data highlights the fact that the cells are gradually differentiating, and the cytoplasmic proteins are migrating to the membrane for the subsequent formation of cellular junctions, so the reduction of the LMR is an index of induction to cell differentiation. Finally, on the twenty-first day, considering that the monolayer of fully differentiated and polarized cells has now been formed and the

formation of tight junctions between the cells has occurred, the reduction of the sample cultures LMR, compared to control, is confirmed. Thus, we can conclude saying that the peptide has proved to be able not only to promote cell differentiation, but also to significantly reduce intestinal permeability.
According to previous studies, we found that un-stimulated Caco-2 cells over-expressed COX-2 and NF-κB and TNF-α-stimulated Caco-2 cells increased 5-LOX expression. NF-κB transcription factor is a master regulator of the inflammatory response and it is essential for the homeostasis of the immune system. NF-κB regulates the transcription of genes, such as LOX and COX-2, which control inflammation (Christian, et al. 2016). Our results demonstrated that MBCP reduced the phosphorylation of NF-κB as well as COX-2 and 5-LOX expression in TNF-α stimulated cells.
Many mechanisms are responsible for the regulation and stability of adherens junction proteins, one of these possibilities could be the AChR activation. Furthermore, previous studies indicate that cholinergic agonists interfere with NF-κB pathways preventing IkBα breakdown and p65 nuclear translocation (Khan, et al. 2015; Rioux and Castonguay 2000). Moreover, cholinergic agonists could prevent gut barrier failure after severe burn injury maintaining intestinal barrier integrity. McGilligan has showed that nicotine decrease Caco-2 permeability by regulating the expression of TJ proteins (McGilligan, et al. 2007). Therefore, we measured changes in localization of β-catenin to determine whether modulation of this protein correlates with the adherens junctions disorganization induced by cholinergic antagonists like atropine and tubocurarine. Confocal microscopy analysis confirmed that both atropine and tubocurarine treatment induced a cytoplasmic accumulation of β-catenin in Caco-2 cells. These detrimental effects induced by cholinergic antagonists were counteracted by MBCP treatment.

7.4.2 MBPC Ameliorates Murine Colitis

The ability of MBCP to restore cell–cell contacts as well as to exert an anti-inflammatory action was subsequently evaluated *in vivo* by using the mice model of DNBS-induced colitis. According to previous studies (Borrelli, et al. 2013; Borrelli, et al. 2015) intracolonic administration of DNBS induced intestinal inflammation associated to an increase of epithelial permeability. Oral MBCP administration was able to reduce intestinal inflammation as demonstrated by the reduction of colon weight colon length ratio (a simple and reliable marker of inflammation and damage), histological alterations, IκBα phosphorylation and of NF-κB activation associated with DNBS administration.

Intestinal permeability plays a crucial role in the development of IBD as well as in the IBD ongoing bowel symptoms (Chang, et al. 2017). Moreover, inflammation reduces barrier integrity and affects the normal intestinal permeability (Bischoff, et al. 2014). Studies on Caco-2 cells have shown that MBCP restored tight junctions altered by TNF-α. Tight junctions are multi-protein complexes that maintain the intestinal barrier while regulating permeability (Edelblum and Turner 2009). Therefore, we investigated the effect of MBPC on intestinal permeability *in vivo*. We adopted an immunofluorescent method through which an orally administered marker (i.e., FITC-conjugated dextran) can be detected in the blood if permeability is impaired. As expected, intestinal permeability increased after DNBS administration and, more importantly, MBCP restored the impaired permeability.

7.4.3 MBPC Normalises Inflammation-Induced Murine Intestinal Hypermotility

It is clinically well established that inflammation in the gut causes debilitating symptoms due to motility disturbances (Brierley and Linden 2014). To investigate the effect of MBCP on intestinal motility, we administered mice croton oil, which is a flogogen agent able to induce hypermotility as a consequence of intestinal inflammation. By using this experimental model, we have shown that MBPC did not affect motility in healthy mice, but normalized the exaggerated intestinal transit caused by the inflammatory insult. The lack of effect of MBPC in control mice is clinically relevant in the light of the observation that constipation is a very common side effect associated with drugs clinically used to reduce intestinal motility (Corsetti and Tack 2015; Rao, et al. 2016).

7.4.4 A novel nutraceutical product ameliorates intestinal permeability in a randomised clinical trial

Encouraging by such promising *in vitro* and *in vivo* results, the final step of the work was the formulation of a novel nutraceutical product for intestinal inflammatory diseases treatment and prevention. It is composed by amino acids corresponding quali-quantitative to the composition of MBCP previously tested. The composition of amino acids mix was: Cys-Lys-Tyr-Val-Thr-Met-Ser in ratio 3:2:1:1:1:1:1.

The choice of test amino acids mix and not MBCP depends on three reasons. The first is that the *in vitro* gastrointestinal digestion of MBCP affects only the first gastrointestinal tract and reaches the colon, so we do not know if after the colon MBCP it breaks down further to release amino acids. The second reason is that the synthesis or isolation of a pure peptide results is a pharmaceutical product rather than a nutraceutical. Finally, *in vitro* studies on the presence of the peptide have not been performed, but the peptide has only

been marked with a chromophore. Thus, we do not know if the pharmacophoric portion belongs to the whole peptide or only to a single portion.

The evaluation of intestinal permeability of enrolled patients has been conduct by measuring the percentage of excretion of the sugars in the urine after 5 h from consumption of a sugar solution containing lactulose and mannitol in 5:1 ratio. A physiological state is defined by an LMR equal or lower than 0.03. Mannitol is a small molecule that use transcellular way to overstep intestinal membrane, so it is able to exploit aqueous porous between cells, while lactulose is an oligosaccharide which use the paracellular way, thus the tight junctions. In this way, mannitol will always overstep the membrane and it will be excreted unchanged in the urine, while the passage of lactulose will be influenced by tight junctions. If there is an alteration of paracellular permeability, lactulose will overstep more than in physiological condition and the lactulose mannitol ratio will be over the limit of 0.03.

Despite it was observed an LMR reduction after 60 days of treatment of 75%, no one of enrolled patients showed a physiological value of LMR. This could be justified by the high baseline LMR value, and the limited length of the clinical trial.

7.5 Conclusions

In conclusion, the data obtained in our study shown that MBCP, a peptide isolated from the buffalo milk after *in vitro* digestion of Mozzarella di Bufala Campana DOP, exerts anti-inflammatory effects both *in vitro* and *in vivo*. This anti-inflammatory effect could be related to its beneficial effects on adherens junctions mainly during an inflammatory process. These results could have an important impact on the therapeutic potential of MBCP in helping to restore the intestinal epithelium integrity damaged by inflammation, thereby reducing the risk of colorectal cancer. Thus, a novel nutraceutical formulation based on the same quali-quantitaive of MBCP was successively tested on humans, showing an improvement of intestinal permeability. This result lead to the hypothesis to suggest MBCP derived nutraceutical as a novel product relevant in intestinal inflammatory diseases.

8 Peschiole (green peaches), an agri-food waste product as a potential source of abscisic acid for hypoglycaemic nutraceutical applications: a clinical trial

8.1 Introduction

2-Cis,4-trans-abscisic acid (ABA) plays a major role in various aspects of plant growth and development, including seed maturation and germination, adaptation to environmental stresses, as well as fruit development (Coombe 1992; Rock and Quatrano 1995); indeed its concentration is higher in an immature stage of the fruit. ABA is also produced and active in humans, playing a key role in the regulation of glucose homeostasis, through different mechanisms of action, as mentioned in chapter 4.1.3. Plasma concentration of ABA (ABAp) increases in healthy subjects after an oral glucose load, stimulating glucose uptake in a manner similar to insulin (Bruzzone, et al. (2012). An impairment of ABAp response after an oral glucose load, instead, occur in T1DM, due to the autoimmune destruction of pancreatic β cells (Bruzzone, et al. (2012), and in T2DM and gestational diabetes (Ameri, et al. 2015), due to an insufficient synthesis or a release dysfunction in response to glucose. Nonetheless, the ABA-induced insulin release depends on the dose. Indeed, a study conducted on rats and humans (Magnone, et al. 2015), where an oral ABA dose between 0.5 and 1 μg per Kg body weight was administered, shows an improved glycaemic profile and low insulin levels after an oral glucose load, compared to controls. Instead, the same test performed with ABA concentrations of 50 mg per Kg body weight caused an increase in insulinemic response, due to GLP-1 stimulation. Hence, low-dose ABA improves glucose tolerance in healthy subjects without increasing insulin and preserving pancreatic β cells, especially useful in subjects with β cell deficiency or with insulin-resistance (T1DM).

Thus, ABA may be a critical factor in maintaining normal glucose tolerance and, therefore, the loss of the physiological response of ABA to glucose could contribute to the pathogenesis of diabetes or prediabetes.

Nutraceutical products indicated for the control of plasma glycemia are already available in the market, even containing ABA. Leading commercial products are represented by the green coffee derivatives, which address their hypoglycemic potential mainly to their content of chlorogenic acids, bioactive compounds widely recognised as able of a positive influence on glycemia both by promoting active plasma glucose consumption and increasing production GLP-1 (Cao, et al. 2015; McCarty 2005). Nevertheless, although its undisputable efficacy, green coffee employed by the nutraceutical industry has to be

subtracted from roasting for the production of the final product destined to the beverage industry.

Peschiole, instead, are a fruit thinning product, a pratice which may interest up to 40% of the entire tree fruit load. This process may lead to a massive agricultural waste product which is generally destined to fertilising or feeding. Occasionally, these waste fruits are recovered to produce an agro-food industry waste product which, in case of hypoglycaemic potential, could be conveniently used as a low or even no-cost source of nutraceutical products. Moreover, such ABA containing products would favour the intake of a human endogenous hormone, contributing to its plasma levels and, thus, to its physiological capacity to modulate glycemia. Thus, the aim of this study was to determine the effects of a novel nutraceutical product based on peschiole, standardised in ABA content, on glycemic and insulinemic responses in a randomised clinical trial.

8.2 Materials and methods

8.2.1 Peschiole collection

Peach samples were collected in June 2018 in Vairano Patenora (Caserta, Italy) at 20-25 days after full bloom, coinciding with fruit thinning stage.

8.2.2 Preparation of peschiole extract and supplement

Peschiole have been extracted with water at 50 °C for 3 hours, the obtained solution has been filtered, centrifuged, and concentrated. Then, the solution has been spray-dried in combination with maltodextrins in a 1:1 ratio, obtaining a fine powder of peschiole extract (PE). With regard to the clinical trial, the PE supplement used in this study consisted of a sachet containing PE microencapsulated with maltodextrins (1 g). This was formulated by the Department of Pharmacy, University of Naples ‘‘Federico II’’ (Naples, Italy).

8.2.3 Characterization of PE

PE (5 g) was treated with 30 ml of methanol (1% acetic acid), homogenised for 5 min by ultra-turrax (T25-digital, IKA, Staufen im Breisgau, Berlin, Germany) and shaken on an orbital shaker (Sko-DXL, Argolab, Carpy, Italy) at 300 rpm for 10 min. Then, the sample were placed in an ultrasonic bath for another 10 min, before being centrifuged at 6000 g for 10 min. The supernatant was collected and stored in darkness, at 4 °C. The pellets obtained were re-extracted, as described above and with another 20 ml of the same mixture of solvents (Riccio, et al. 2017). Finally, the two supernatants obtained were united and filtered under vacuum. An aliquot was filtered through RC 0.45 μm membrane, and 20 μl of the filtrate were injected into an HPLC-DAD system.

8.2.3.1 HPLC-DAD-MS analysis

The chromatographic analyses were performed according to previous authors with some modification (Bosco, et al. 2013). Analyses were performed on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD), provided with a diode array detector (DAD), coupled to an Advion Expression mass spectrometer (Advion Inc., Ithaca, NY) equipped with an Electrospray (ESI) source. Mass spectra were recorded in negative SIM mode. The capillary voltage was set at 150 V, the spray voltage was at 3 kV, the source voltage offset was at 25 V and the capillary temperature was set at 300 °C. The column selected was a Kinetex® C18 column 100Å (250 mm x 4.6 mm i.d., 5 μm) (Phenomenex, Torrance, CA). The mobile phase was (A) 0.05% formic acid, and (B) acetonitrile. The column temperature was maintained at 30 °C. Separation was carried out by gradient elution with a constant flow rate of 1 ml/min. The gradient program was as follows: 5% B (0-3 min), 5−75% B (3−24 min), 75-5% B in one minute and a re-equilibration of 10 min. An injection volume of 20 μl was used for each analysis. Peak identification was confirmed with HPLC-MS experiment. Calibration curve of ABA, detected at 265 nm, was performed with standard samples prepared in methanol, freshly on a daily basis over a concentration range of 0.1-1.0 μg/μl with six different concentration levels.

8.2.4 Study population

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled in July 2019. Patients aged 51-77 years were eligible for enrolment if they had the following values of serum parameters at baseline: glucose ≤ 200 mg/dl, insulin < 13.16 μU/ml and glycated haemoglobin (HbA1c) < 108 mmol/mol. Exclusion criteria were as follows: smoking, hepatic disease, renal disease, heart disease, family history of chronic diseases, intense physical exercise (>10 h/week), pregnant women, women suspected of being pregnant, women who hope to become pregnant, breastfeeding, birch pollen allergy and blood donation less than 3 months before the study. The subjects received oral and written information regarding the study before they gave their written consent. Protocol, letter of intent of volunteers and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (prot.70128, 15 May 2017) and carried out in accordance with the Helsinki Declaration of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a randomised, double-blind, monocentric, placebo-controlled trial. The study duration was 20 weeks: the group underwent 4 weeks of run-in,

followed by 12 weeks of nutraceutical treatment, and 4 weeks of follow-up. Both the examinations and the study treatment were performed in an outpatient setting. Clinic visits and blood sampling were performed at weeks 0, 8, 12 and 16. Clinic visits and blood sampling to test hepatic and renal toxicity, were performed after 12 h of fasting at weeks 0, 4, 8, 12 and 16. Subjects were informed not to drink alcohol or perform hard physical activity 48 h before blood sampling. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected in 10-ml EDTA-coated tubes (Becton-Dickinson, Plymouth, United Kingdom) and plasma was isolated by centrifugation (20 min, 2200 g, 4 C°). All samples were stored at -80° C until analysis. Plasma glucose and HbA1 levels were determined using commercially available kits from Diacron International (Grosseto, Italy). Plasma insulin concentrations were measured using an insulin enzyme-linked immunoassay (Insulin ELISA kit, ALPCO®, Salem, NH, USA).

In addition to these five meetings, five standardised telephone interviews were performed starting from the first meeting to verify compliance and increase protocol adherence. In particular, these interviews reminded patients to complete their intake checking table for the intervention study and to record any discontinuation or adverse events they might have experienced in the meantime (which were also documented regularly on the case report forms during each telephone and clinic visit). All patients underwent a standardised physical examination, assessment of medical history (for up to 5 years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. At each clinic visit, patients had to complete three self-administered questionnaires on quality-of-life aspects, and their diaries were checked for data completeness and quality of documentation to ensure patient comprehension of the diary items.

8.2.5 Study treatment

The group of 40 patients (24 men and 16 women) was randomly divided into two subgroups (each one of 20 subjects). All subjects assuming sachets (1 g each one) were instructed to take two sachets for meal (breakfast, lunch, and dinner), for a total of 6 sachets/day, corresponding to 30 μg ABA for meal. Treatment group take sachets of PE, while placebo group took identically appearing sachets containing only maltodextrins, for 12 weeks.

8.2.6 Randomization, concealment and blinding

A total of 40 eligible patients were randomly assigned to two groups to receive the treatment or placebo. Supplements and placebo were coded with different colours and given in random order. The code was not broken until all analyses were completed and the results were analysed statistically. If a patient dropped out before receiving supplement, he or she was replaced by the next eligible patient enrolled at the same centre. The concealed allocation was performed by an Internet-based randomization schedule, stratified by study site. The random number list was generated by an investigator without clinical involvement in the trials. Patients, physicians, lab technicians and trial staff (data analysts, statisticians) were blind to treatment allocation.

8.2.7 Study outcomes and data collection

8.2.7.1 Primary and secondary efficacy outcomes.

The primary endpoints measured were the variations of glycaemia, insulin, HbA1c and HOMA-IR index, while key secondary outcomes collected during clinic visits were measurements of blood pressure and heart rate. All raw patient ratings were evaluated in a blinded manner at the site of the principal investigator. The decision process was performed according to a consensus document (unpublished standard operating procedure) before unblinding to define conclusive primary and secondary efficacy data from a clinical perspective.

8.2.7.2 Safety

We assessed safety from reports of adverse events as well as laboratory parameters concerning hepatic and renal function, vital signs (blood pressure, pulse, height and weight), and physical or neurological examinations. Safety was assessed over the entire treatment period at weeks 0, 8, 12, 16 and 20 including adverse events occurring in the first 3 weeks after cessation of treatments.

8.2.8 Statistic

8.2.8.1 Methodology

During the trial, it became apparent that dropouts and incomplete diary documentation created missing data that could not be adequately handled by the intended robust comparison. To deal with the missing data structure, we used a negative binomial, generalised linear mixed effects model (NB GLMM) that not only yields unbiased parameter estimates when missing observations are missing at random (MAR) (Little and Rubin 2019), but also provides reasonably stable results even when the assumption of MAR is violated (Molenberghs, et al. 2004; O'Kelly and Ratitch 2014). Patients who did

not provide any diary data (leading to zero evaluable days) were excluded from the MAR based primary efficacy analysis, according to an “all observed data approach” as proposed by White, et al. (2012). This approach is statistically efficient without using multiple imputation techniques (Carpenter and Kenward 2007). Data retrieved after withdrawal of randomised study treatment were also included in the analysis.

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student’s t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed by using Cochran’s test ($p<0.1$). The I^2 statistic was also calculated, and $I^2>50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. Percent change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% CIs, p-values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration, total polyphenols dose, and Jadad score. Treatment effects were analysed using PROC MIXED with treatment and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in serum parameters and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P < 0.05$).

8.2.8.2 Analysis sets

The full analysis set population included all randomised patients and patients who did not fail to satisfy a major entry criterion. We excluded patients who provided neither primary nor secondary efficacy data from efficacy analyses. The per protocol set consisted of all patients who did not substantially deviate from the protocol; they had two characteristics. First, this group included patients for whom no major protocol violations were detected (e.g., poor compliance, errors in treatment assignment). Second, they had to have been on treatment for at least 50 days counting from day of first intake (completion of a certain

prespecified minimal exposure to the treatment regimen). Hence, patients who prematurely discontinued the study or treatment were excluded from the per protocol sample.

8.2.9 Patient involvement

No patients were involved in setting the research question or the outcome measures, nor were they involved in developing plans for participant recruitment or the design and implementation of the study. There are no plans to explicitly involve patients in dissemination. Final results will be sent to all participating sites.

8.3 Results

8.3.1 HPLC-MS analysis of PE

The quantitative analysis of ABA content of PE prepared for this study was assessed by HPLD-DAD, while the qualitative identification of ABA peak was assessed by LC-MS analysis. PE contains 15 µg ABA/g of dry weight. Figure 21 shows the HPLC chromatogram of PE analysis.

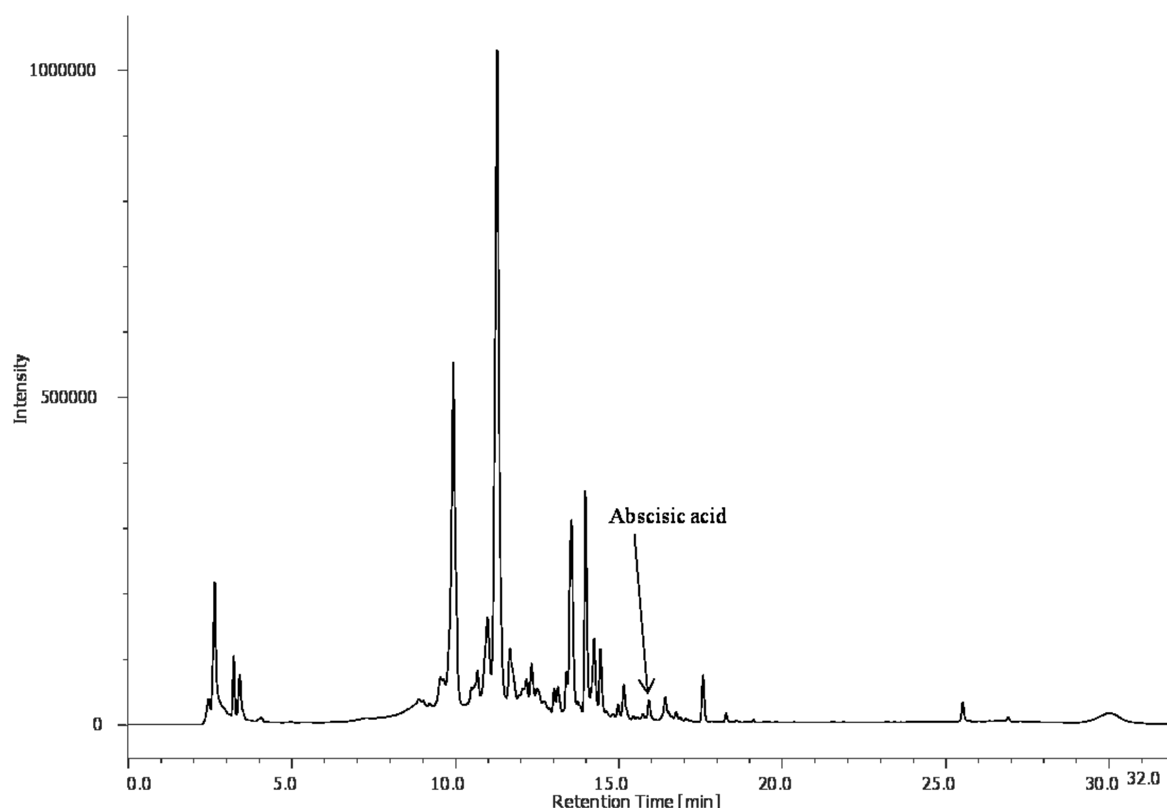

Fig. 21 *HPLC-DAD chromatogram of ABA in peschiole extract*

8.3.2 Enrolment and subject attrition

Patients were enrolled in June 2019. A total of 60 patients were screened for eligibility; 20 patients (33.3%) did not pass the screening stage; 40 patients were randomised. The most

common reason was that patients did not meet the inclusion criteria regarding values of serum parameters at baseline (n=6), followed by concerns about the protocol (n=5), and other reasons (n=4). Some fulfilled exclusion criteria (n=5). Overall, 20 patients were assigned to the group of intervention study: they underwent a run-in period during the first 4 weeks before the treatment period of 12 weeks. Follow-up period last other 4 weeks. Fig. 22 shows the flow of participants through the trials together with the completeness of diary information over the entire treatment period. No patient prematurely terminated study participation before allocation to treatment. Fig. 22 follows the CONSORT PRO reporting guideline (Raederstorff, et al. 2015) and reveals that within the assessment period, the following percentage of patients for each group provided data for the primary endpoint: treatment group 89.4% (17 of 19 patients); placebo group 82.3% (14 of 17 patients). In each group, a few patients did not submit any diaries, giving no specific reason for this. Completeness of the patient diaries did not differ between the treatment groups.

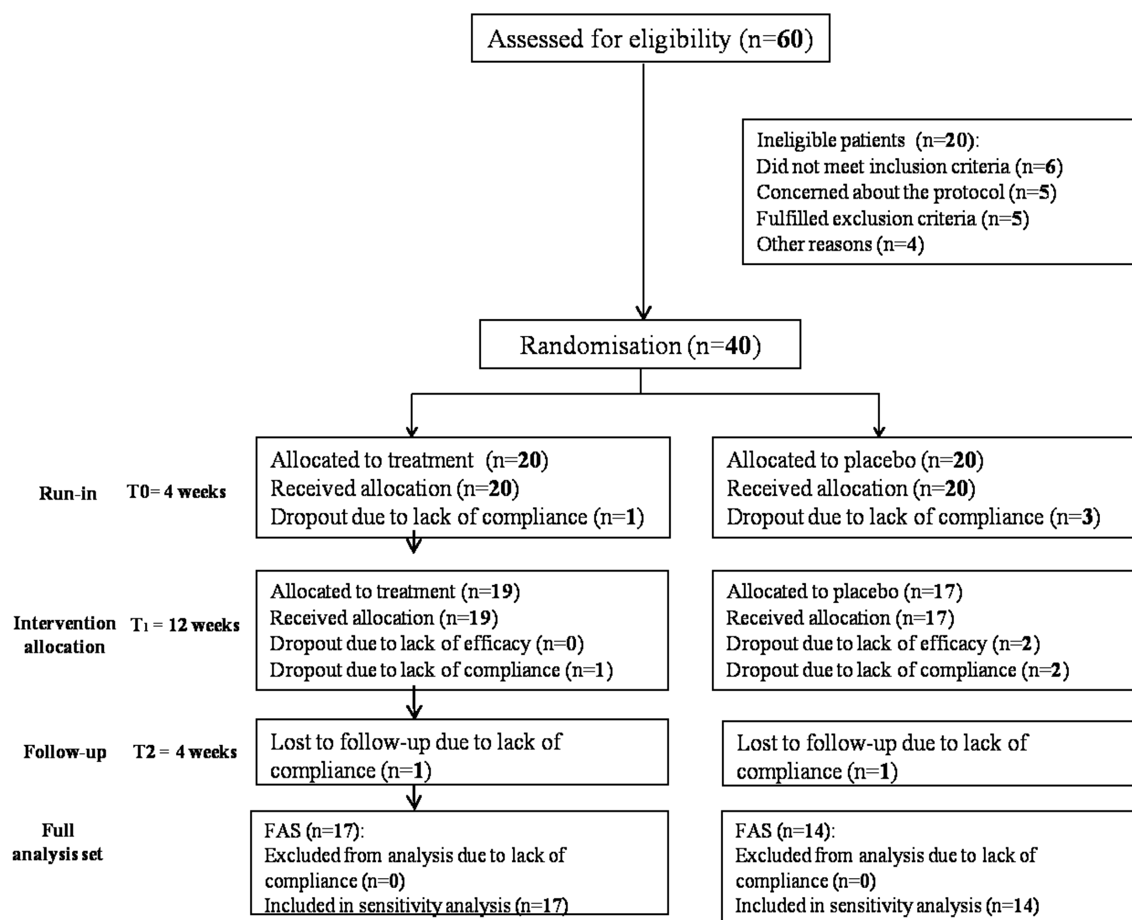

Fig.22 *Study flowchart, according to the consolidated standards of reporting trials (CONSORT).*
The diagram shows enrolment and primary efficacy endpoints based on patient diaries, from pre-screening to data collection; and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period.
FAS=full analysis set.

8.3.3 Participants' baseline characteristics

Table 14 shows the demographic and clinical characteristics assessed at the baseline visit of all 40 patients randomised. Overall, about half of the randomised patients were female; the total age range was 57-77 years. The groups were well balanced for demographics and clinical factors.

Table 14 *Baseline characteristics of intention to treat sample according to study treatment.*

Treatment	
Characteristics	
Demographics	
Age (years)	64.2 ± 10.3
Male sex (No %))	11 (60%)
White ethnicity (No (%))	20 (100%)
Clinical parameters	
Glucose (mg/dl)	137.11 ± 22.95
Insulin (μU/ml)	7.64 ± 2.21
HbA1c (mmol/mol)	72 ± 1.27
HOMA-IR index	2.63 ± 1.12
Placebo	
Characteristics	
Demographics	
Age (years)	65.1 ± 9.8
Male sex (No %))	13 (65%)
White ethnicity (No (%))	20 (100%)
Clinical parameters	
Glucose (mg/dl)	102.06 ± 25.65
Insulin (μU/ml)	6.93 ± 1.63
HbA1c (mmol/mol)	71 ± 1.28
HOMA-IR index	1.81 ± 0.87

Values are means ± SD (n=5). Results were significantly different at a level of P=.001.

8.3.4 Primary efficacy outcomes measures

No significant variations of plasma glucose, insulin and HbA1c with respect to the baseline values, were registered in subjects at the end of the placebo period (Table 15). Analysing results regarding the variation of glycaemia, insulin response, HOMA-IR index and HbA1c values at the end of the intervention period (90 days), we can observe a significant decrease of the mean values of glycaemia (-34.2%, $P<0.0001$), insulin (-33.63%, $P<0.001$), HOMA-IR index (-56.89%, $P<0.0001$), and Hb1Ac (-17.76%, P<0.001).

Table 15 *Effects of PE formulation on plasma glycaemia, insulin response, HbA1c and HOMA-IR index values*

		Treatment group	Δ%
Glucose (mg/dl)	T_0	137.11 ± 22.95	
	T_{30}	106.04 ± 35.82	-22.66 *
	T_{60}	100.3 ± 16.34	-26.85 *
	T_{90}	90.22 ± 16.47	-34.2 *
Insulin (µU/ml)	T_0	7.64 ± 2.21	
	T_{30}	5.54 ± 1.5	-27.53 *
	T_{60}	5.68 ± 0.62	-25.7 *
	T_{90}	5.07 ± 1.01	-33.63 *
HOMA-IR	T_0	2.63 ± 1.12	
	T_{30}	1.46 ± 0.64	-44.56 *
	T_{60}	1.41 ± 0.27	-46.61 *
	T_{90}	1.13 ± 0.31	-56.89 *
HbA1c (mmol/mol)	T_0	71 ± 1.27	
	T_{30}	59 ± 0.3	-12.24
	T_{60}	55 ± 0.12	-15.94
	T_{90}	52 ± 0.45	-20.18 *

Values are means ± SD (n=5).
T_0: 1st day of treatment; T_{30}: 30th day of treatment; T_{60}: 60th day of treatment; T_{90} 90th day of treatment.
* Significantly different from baseline at $P < .01$ (PROC MIXED).

8.3.5 Safety issues. study strength and limitations

No adverse effects of the treatment were reported. Other safety assessments, such as vital signs, blood pressure and heart rate were all periodically monitored, and baseline values did not change substantially during and at the end of the trial.

The major strengths of the clinical trial herein presented reside in the originality of the study and in the evaluation of the treatment effects in prediabetic or diabetic patients. Conversely, the main limitations of our study include the short-term assessment for the treatment of a chronic condition, a limited cohort of enrolled patient, the choice of exclusively white race, and the wide age range due to the availability of such individuals at the stage of the recruitment.

8.4 Discussions

Our HPLC data regarding quantification of ABA in PE confirmed that this hormone is mainly concentrated in the earliest development stages of fruits (Magnone, et al. 2015; Sun, et al. 2012; Zhao, et al. 2012). Thus, the product of the fruit thinning practice, in our case, peschiole, can be regarded as a good source of ABA.

Results of the clinical trial indicate that the chronic intake of the ABA-containing nutraceutical (30 µg for meal), for 90 days in diabetic and prediabetic patients, significantly reduced glycaemia, insulin response, HbA1c and HOMA-IR index. The present study originates from a previous finding that low-dose oral ABA (1µg/kg body weight) improves glucose tolerance, without increasing insulin in humans and rats (Magnone, et al. 2015). Instead, higher oral dose ABA (100 mg/kg body weight) showed a significant decrease in glycaemia levels, but also an elevation of insulin. These results suggest that the main target of low-dose ABA *in vivo* is the stimulation of glucose uptake in muscle, causing a reduction of blood glucose levels and consequently of insulin secretion. Indeed, *in vitro* studies have demonstrated a direct, insulin-independent effect of ABA on GLUT4 expression and membrane translocation in murine myoblasts (Bruzzone, et al. 2012).

Insulin-independent stimulation of glucose transport in the skeletal muscle may account for the glycemia-lowering effect of ABA observed in chronic treatment. Noteworthy, the prolonged stimulation of β-cells to release insulin under conditions of chronic hyperglycaemia contributes to the eventual demise of the β-cell population (Muoio and Newgard 2008). For this reason, antidiabetic drugs capable of lowering glycaemia without increasing insulin values are highly desirable. Hence, the administration of ABA supplements was to be proposed as an aid to improving glucose tolerance in diabetic or prediabetic subjects who are deficient in or resistant to insulin, in order to support the survival and function of pancreatic β cells.

Although ABA supplements have been previously tested on postprandial glycaemia and insulin response in rat and human studies (Atkinson, et al. 2019; Magnone, et al. 2018), showing promising results, our innovation is the formulation of a nutraceutical product deriving from waste product of the agri-food industry, thus recovering a product intended for disposal.

The dose of ABA administered in the clinical study is not attainable from a vegetal-rich diet, although ABA is present in most leafy vegetables, seeds, legumes, and fruits (Zocchi, et al. 2019). ABA intake from these sources would range from approximately 160–260 µg ABA per day (on average fruits contain 0.62 µg ABA/g and vegetables contain 0.29 µg ABA/g (Zocchi, et al. 2017). The American Heart Association 2020 Strategic Goals recommend ≥ 4.5 servings/day of fruits and vegetables (Lloyd-Jones, et al. 2010), which is estimated to provide ≥ 297 µg of ABA per day (Zocchi, et al. 2017). However, only 8% of the US adult population meets these dietary recommendations (Rehm, et al. 2016),

suggesting that the majority of the population is consuming diets low in ABA, which could impact overall health outcomes.

Thus, intake of a food supplement containing the appropriate amount of ABA is required to achieve this dose. Absorption of ingested ABA has been already documented (Magnone, et al. 2015), probably occurring by simple diffusion of the protonated molecule at pH values below the pKa of ABA (4.6), such as those present in the stomach. The long half-life of ABA in the bloodstream, inferred from the elevated ABAp levels observed in humans several hours after intake of an ABA-rich extract (Magnone, et al. 2015), is likely due to binding of ABA to plasma proteins. Indeed, ABA binding to fatty-acid-free human albumin has been observed *in vitro* (Magnone, et al. 2015) and is reminiscent of the behaviour of steroid and thyroid hormones, which share a relatively long half-life.

8.5 Conclusions

Our experimental data indicated peschiole as a good source of ABA, in particular 15 µg/g of PE dry weight. The results from the clinical trial presented here provide a strong rationale for the supplementation of human diet with a novel nutraceutical formulation containing ABA as a means to correct the initial derangement of the glucose homeostasis (blood glucose, insulin, Hb1Ac and HOMA-IR index) which are symptomatic and pathogenetic of prediabetes and diabetes. Therefore, peschiole may be regarded as a valuable agri-food industry waste product for the formulation of nutraceutical remedies indicated for the control of plasma glycaemia.

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