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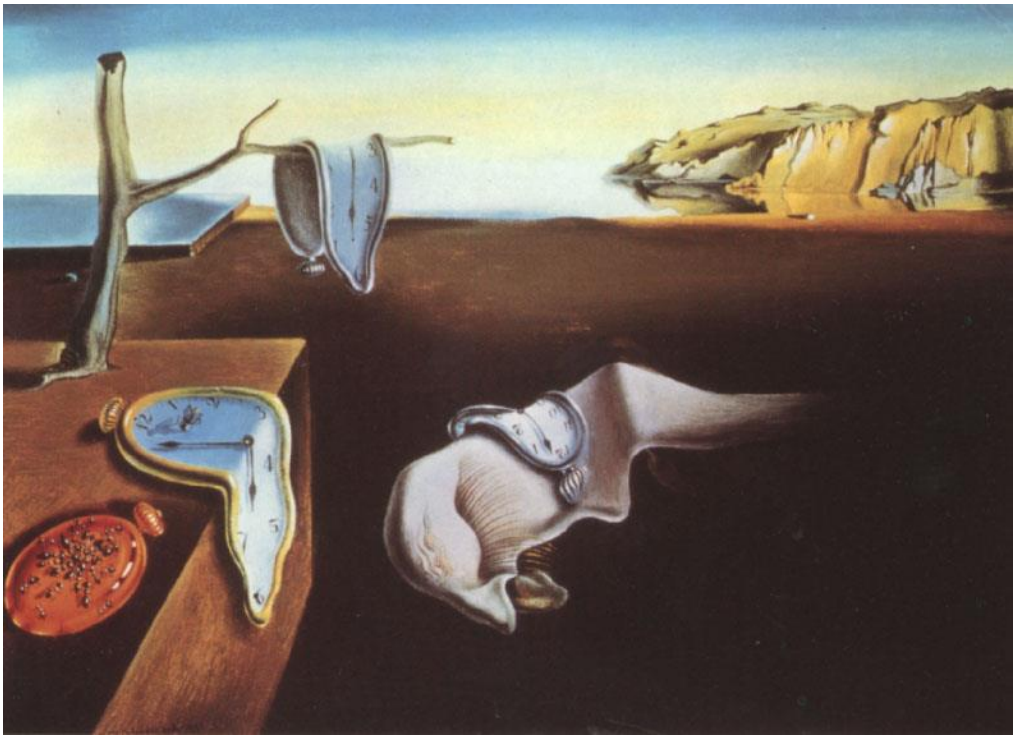
Healthy eating as a strategy to achieve successful ageing: focus on Mediterranean diet and functional foods



Anna Aiello

UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di ricerca in Medicina Molecolare e Biotecnologie
Dipartimento di Biopatologia e Biotecnologie Mediche
SSD Med/04

HEALTHY EATING AS A STRATEGY TO ACHIEVE SUCCESSFUL AGEING: FOCUS ON MEDITERRANEAN DIET AND FUNCTIONAL FOODS

IL DOTTORE
ANNA AIELLO

IL COORDINATORE
PROF. CALOGERO CARUSO

IL TUTOR
PROF.SSA GIUSEPPINA CANDORE

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“Let food be your medicine and medicine be your food”
Hippocrates

“Remember to look up at the stars
and not down at your feet…And
however difficult life may seem, there is
always something you can do,
and succeed at”
S.W. Hawking

“Non ho paura di morire, è un
compito biologico di ogni essere vivente,
per lasciare spazio a nuove generazioni.
Da medico mi appassionano gli studi
sulla longevità ma il nostro vero obiettivo deve
essere non solo vivere più a lungo, ma
godere del tempo guadagnato, in uno
stato di salute che consenta una vita attiva
del corpo e soprattutto della mente”
U. Veronesi

TABLE OF CONTENTS

Abstract of papers produced during PhD course and relevant to this thesis

1. **Aiello A**, Dara Guccione G, Accardi G, Caruso C. What olive oil for healthy ageing? Maturitas. 2015;80:117-8.

Excerpt

It is widely accepted that olive oil plays a key role in explaining part of the health benefits of the Mediterranean Diet. But, does an olive oil recommended for healthy ageing exist? Do all olive oils have the same properties? Olive oil is a complex mixture of over 200 compounds whose composition depends on many factors, such as geographical origin, weather and irrigation, and cultivation techniques. To be considered as extra virgin, an olive oil must have free acidity, expressed as oleic acid, of no more than 0.8%, and it must be obtained by mechanical processing, or "cold pressing", that preserves polyphenol fraction. Extra virgin is probably the variety of olive oil to be recommended for healthy ageing due to its greater amount of polyphenols, with antioxidant and anti-inflammatory properties, and oleic acid, which is claimed to increase the resistance of low-density lipoprotein to oxidation and, consequently, reduces the risk of atherosclerosis.

2. Accardi G, **Aiello A**, Caruso C. Obesità, microbiota e aging (Obesity, microbiota, and aging). In "La medicina dell'aging e dell'anti-aging". Edra. 2016;251-265.

Excerpt

Obesity is one of the most prevalent health issues of our time. More and more deaths are caused worldwide by excessive weight, mainly determined by an imbalance between energy intake and energy expenditure. While modern eating habits and ever increasingly sedentary lifestyles are major contributory

factors, a link has recently emerged between obesity and the composition and functionality of gut microorganisms, probably because different kinds of diets are associated with differences in the composition of gut microbiota. Moreover, several research groups demonstrated the contribution of gut microbial communities to old people and their role on healthy ageing owing to their strong impact on human metabolism and immunology. Indeed, changes with age in specific bacterial genera and species have been identified, with a considerable inter-individual variation that continues into old age.

3. Accardi G, **Aiello A**, Gambino CM, Virruso C, Caruso C, Candore G. Mediterranean nutraceutical foods: Strategy to improve vascular ageing. Mech Ageing Dev. 2016;S0047-6374:30011-2.

Abstract

Ageing is characterized by a decline in all systemic functions. A greater susceptibility to apoptosis and senescence may contribute to proliferative and functional impairment of endothelial progenitor cells. These play an important role in neo-angiogenesis and endothelial repair. Vascular ageing is associated with changes in the structure and functions of vessels' walls. There are many possible causes of this damage. Without doubt, inflammation and oxidative stress play a fundamental role in the pathogenesis of endothelial dysfunction, commonly attributed to a reduced availability of nitric oxide. Inflammageing, the chronic low-grade inflammation that characterizes elderly people, aggravates vascular pathology and provokes atherosclerosis, the major cardiovascular disease. Nutraceutical and molecular biology represent new insights in this field. Indeed, the first could represent a possible treatment in the prevention or delay of vascular ageing; the second could offer new possible targets for

potential therapeutic interventions. In this review, we pay attention to the causes of vascular ageing and on the effects of nutraceuticals on it.

4. Accardi G*, **Aiello A***, Gargano V, Gambino CM, Caracappa S, Marineo S, Vesco G, Carru C, Zinellu A, Zarcone M, Caruso C, Candore G. Nutraceutical effects of table green olives: a pilot study with *Nocellara del Belice* olives. Immun Ageing.2016;13:11.

Abstract

Background. The aim of this study was to analyse the nutraceutical properties of table green olives *Nocellara del Belice*, a traditional Mediterranean food. The key elements of the Mediterranean Diet are olives and extra virgin olive oil, common to all Mediterranean countries. Olive oil is the main source of fat and can modulate oxidative stress and inflammation, whereas little is known about the role of olives. Moreover, emerging evidences underline the association between gut microbiota and food as the basis of many phenomena that affect health and delay or avoid the onset of some age-related chronic diseases.

Methods. In order to show if table green olives have nutraceutical properties and/or probiotic effect, we performed a nutritional intervention, administering 12 table green olives/day for 30 days to 25 healthy subjects (mean age 38.3). We carried out anthropometric, biochemical, oxidative stress and cytokine analyses at the beginning of the study and again at the end. In addition, we also collected fecal samples to investigate the possible variation of concentration of Lactobacilli, after the consumption of the olives.

Results. Our results showed a significant variation of one molecule related to oxidative stress, malondialdehyde, confirming that *Nocellara del Belice* green olives could have

an anti-oxidant effect. In addition, the level of interleukin-6 decreased significantly, demonstrating how this food could be able to modulate the inflammatory response. Moreover, the reduction of fat mass with an increase of muscle mass is noteworthy, as it suggests a possible effect of long term assumption of table olives on body mass variation. No statistically significant differences were observed in the amount of Lactobacilli, although a trend towards an increased concentration of them at the end of the intervention could be related to the nutraceutical effects of olives.
Conclusion. These preliminary results suggest a possible nutraceutical effect of daily consumption of green table olives *Nocellara del Belice*. To the best of our knowledge, this is the first study performed to assess nutraceutical properties of this food. Of course, it is necessary to verify the data in a larger sample of individuals to confirm their role as nutraceuticals.
*contributed equally

5. **Aiello A**, Accardi G, Candore G, Gambino CM, Mirisola M, Taormina G, Virruso C, Caruso C. Nutrient sensing pathways as therapeutic targets for healthy ageing. Expert Opin Ther Targets. 2017. *In revision.*

Abstract
Introduction: In the present paper, the authors have discussed anti-ageing strategies which aim to slow the ageing process and to delay the onset of age-related diseases, focusing on nutrient sensing pathways (NSPs) as therapeutic targets. Indeed, several studies have already demonstrated that both in animal models and humans, dietary interventions might have a positive impact on the ageing process through the modulation of these pathways.
Areas Covered: Achieving healthy ageing is the main challenge of the 21th century because the lifespan is increasing,

but not in tandem with good health. The authors have illustrated different approaches that can act on NSPs, modulating the rate of the ageing process.
Expert Opinion: Humanity's lasting dream is to reverse or, at least, postpone ageing. In recent years, increasing attention has been devoted to anti-ageing therapies. The subject is very popular among the general public, whose imagination runs wild with all the possible tools to delay ageing and to gain immortality. Some approaches discussed in the present review should be able to substantially slow down the ageing process, extending our productive, youthful lives, without frailty.

6. Baldassano S, Buscemi S, **Aiello A**, Accardi G, Caruso C, Vasto S. β-Glucans and Post Prandial Satiety: the Role of Intestinal Hormones in Healthy Volunteers. Int J Immunopathol Pharmacol. *Submitted*.

Abstract

Summary: Recent interest in intestinal hormones has risen with the idea that they modulate glucose tolerance and food intake through a variety of mechanisms, and such hormones like peptide YY (PYY), ghrelin, glucagon-like peptide (GLP)-1 and 2, and cholecystokinin (CKK) are therefore excellent therapeutic candidates for the treatment of diabetes and obesity. Furthermore, in the recent years, multiple studies suggest that the microbiota is critically important for normal host functions, while impaired host microbiota interactions contribute to the pathogenesis of numerous common metabolic disorders. In this study, we considered the nutraceutical effects of β-glucans added to pasta at the concentration of 6g\100g. Ten participants have been recruited and hematochemical analyses and intestinal hormones tests have been performed before and after 30 days of pasta intake. Stool specimens have been studied for *Lactobacillus Fermentum*, *Lactobacillus*

acidophilus, *Lactobacillus salivarius*, *Bifidobacterium longum*, and *Enterococcus faecium* presence before and after 30 days of nutritional intervention. After 30 days of regular intake of pasta enriched by β-glucans results have been evaluated. In conclusion, pasta prepared from barley flour enriched with β-glucans at 6% exhibit promising responses on glucose metabolism, on intestinal hormones responses and on microbiota modification.

7. **Aiello A**, Accardi G, Candore G, Carruba G, Davinelli S, Passarino G,Scapagnini G, Vasto S, Caruso C. Nutrigerontology: a key for achieving successful ageing and longevity. Immun Ageing. 2016;13:17.

Abstract

During the last two centuries the average lifespan has increased at a rate of approximately 3 months/year in both sexes, hence the oldest old people are becoming the section of the population with the fastest growth in Western World. Although the average life expectancy is increasing dramatically, the healthy lifespan is not increasing at the same pace. This underscores the importance of studies on the prevention of age-related diseases, in order to satisfactorily decrease the medical, economic and social problems associated to advancing age, related to an increased number of individuals without autonomy and affected by invalidating diseases. In particular, data from experimental studies in model organisms have consistently shown that nutrient signalling pathways are involved in longevity, affecting the prevalence of age-related loss of function, including age-related diseases. Accordingly, nutrigerontology is defined as the scientific discipline that studies the impact of nutrients, foods, macronutrient ratios, and diets on lifespan, ageing process, and age-related diseases. To discuss the potential relevance of this new science in the

attainment of successful ageing and longevity, three original studies performed in Sicily with local foods and two reviews have been assembled in this series.The data clearly demonstrate the positive effects of nutraceuticals, functional foods and Mediterranean Diet on several biological parameters. Indeed, they could represent a way to prevent many age-related diseases, and make possible, if not a solution for this social plague, at least a remedy to alleviate the struggles it causes. Thus, the possibility to create a dietary pattern, based on the combined strategy of the use of both nutraceuticals and functional foods should permit us to create a new therapeutic strategy, based not only on a specific bioactive molecule or on a specific food but on an integrated approach that, starting from the local dietary habits, could pave the way for a "nutrafunctional diet" applicable worldwide.

List of abbreviations

AGEs advanced glycation end-products
AMKT AMP-activated protein kinas
AOP aglicone oleuropein paracetylated
ARE antioxidant response element
BMI body mass index
CALERIE Comprehensive Assessment of the Long-term Effects of Reducing Intake of Energy
CHDs coronary heart diseases
CLA conjugated linoleic acid
CR caloric restriction
CVDs cardiovascular diseases
DMSO dimethyl sulfoxide
DR dietary restriction
EDTA ethylene diamine tetra acetic acid
EPCs endothelial progenitor cells
EVOO extra virgin olive oil
FBS fetal bovine serum
FMD fasting mimicking diet
FOXO fork head box O
GI glycaemic index
GIT gastro-intestinal tract
GLP glucagon like peptide
HDL high density lipoprotein
HEPES hydroxyethyl-1-piperazineethanesulfonic acid
HP hydrossytyrosol paracetylated
IDL intermediate density-lipoprotein
IF intermittent fasting
IGF insulin-like growth factor
IL interleukin
IMIBIC Instituto Maimónides de Investigación Biomédica de Córdoba

LDL low-density lipoprotein
MDA malondialdehyde
MDP MedDiet pyramid
MedDiet Mediterranean Diet
mTOR mammalian target of rapamycin
MUFA mono unsaturated fatty acid
NF-κB kappa-light-chain-enhancer of activated B cells
NO nitric oxide
Nrf2 nuclear factor-E2-related factor 2
NSPs nutrient-sensing pathways
OFI *opuntia ficus indica*
PBMCs peripheral blood mononuclear cells
PF prolonged fasting
PON paraoxonase
PR protein restriction
PSH protein SH
PUFA poly unsaturated fatty acid
PYY peptide YY
ROS radical oxygen species
RPMI Roswell Park Memorial Institute
SA successful ageing
SCFA short-chain fatty acids
SNPs single nucleotide polymorphisms
TBARS thiobarbituric acid reactive substances
TC total cholesterol
TFs transcription factors
TNF tumor necrosis factor
UA unsuccessful ageing
VLDL very low-density lipoprotein
WHO world health organization

I. Introduction: Healthy eating for healthy ageing

People want to live longer. To date, it is impossible not to grow older, but it is desirable to get older in good health, avoiding age-related disabilities. To do this, it is necessary to have a healthy lifestyle which can limit the damage caused by the environmental hazards (radiations, exhaust fumes, heavy metals) that face us each day.

Human beings are always in search of the elixir of long life and, in recent years, science has made great strides. The ageing studies and, in particular, the research of determinant factors for successful ageing and longevity, are steadily growing (*Longo et al., 2015*). However, although life expectancy in western countries has increased approximately 3 months/year in both sexes in the past decade, following improved cares and diagnosis, the years lived with disabilities and diseases have not decreased (*Oeppen et al., 2002*). Therefore, the continuous increase of lifespan does not go hand in hand with the increase of healthy lifespan, the so-called "health-span" (*Kolovou et al., 2014*; *Mercken et al., 2012*). Therefore, the ongoing and future studies ought not only help to increase longevity, but also to promote what is known as "active life expectancy", that is the time in later life spent free

of disability. This is true also if we are to satisfactorily decrease the medical, economic and social problems associated with advancing age, related to an increased number of individuals without autonomy and affected by invalidating diseases (*Katz et al., 1983*).

In humans, healthy ageing and longevity are modulated by a fortunate interaction between genetic and environmental factors (**Figure 1**). Regarding the latter, physical activity and healthy dietary habits are the most important modifiable factors that can affect the maintenance of a healthy ageing phenotype (*Dato et al., 2013*). In particular, nutrition is a daily process by which living organisms get food to gather energy and nutrients to live. Good nutrition plays a significant role in determining the well-being of older people, and in delaying and reducing the risk of contracting diseases. Eating too few fruits and vegetables is, for example, responsible for close to three million deaths worldwide every year. In addition, the intake of dietary fat seems to be associated with various cancers. Nutritionally unbalanced diets are often associated with diabetes and with the risks of developing coronary heart diseases (CHDs). It was seen that children that follow a balanced diet with lots of fruits and vegetables are likely to

continue eating healthily into adulthood. On the contrary, parents who eat too much processed food with high levels of salt, sugar and fat tend to pass those habits onto their own children (*Kiefte-de Jong et al., 2014*).

Nowadays, the findings that diet can improve successful ageing have opened new and interesting suggestions for anti-ageing medicine (*Fontana et al., 2010; Partridge et al., 2010*). Accordingly, a new science, called "nutrigerontology," was born. Nutrigerontology is defined as the scientific discipline that studies the impact of nutrients, foods, macronutrient ratios and diets on lifespan, the ageing process and molecular pathways, and age-related diseases (*Aiello et al., 2016a; Verburgh, 2015*). In fact, when the biogerontologists speak of a "healthy diet", that can slow the ageing process, they are also referring to the modulation of intracellular signaling pathways, the so-called nutrient-sensing pathways (NSPs), that play a fundamental role in healthy ageing and longevity (*Davinelli et al., 2012*). As it will be explained in this thesis, NSPs are activated by nutrients, such as carbohydrates or proteins, that trigger signals which result in a downstream activation of some genes involved in ageing process. An excessive intake of nutrients can accelerate these events and increases the risk of

age-related diseases. On the contrary, healthy diets (those that do not overstimulate NSPs) reduce this risk, promoting successful ageing and longevity (*Aiello et al., 2016 b. In press*).

Although the complex relationship between nutrition, the ageing process and healthy ageing is not completely understood in humans, specific dietary changes, such as dietary restriction (DR), the reduction of glycaemic and protein intake or the elimination of trans and saturated fats, the increased intake of omega-3, vitamins, micronutrients and antioxidants, can help to minimize the inflammageing, phenomenon that characterizes ageing (see **I.I**). Similarly, appropriate intake of specific foods, the so-called "functional foods", may confer health benefits, influencing the maintenance of immune homeostasis, and contributing, directly, to the reduction of inflammation and metabolic disorders (*Dato el al., 2016*; *Kiefte-de Jong et al., 2014*) (see **I.III.II**).

The majority of the evidence that links nutrition and healthy ageing is derived from epidemiological studies. On the contrary, nutritional interventions, the typical model approaches used to analyse the role of specific foods and diets on healthy ageing, are needed to support the development of personalised treatment plans.

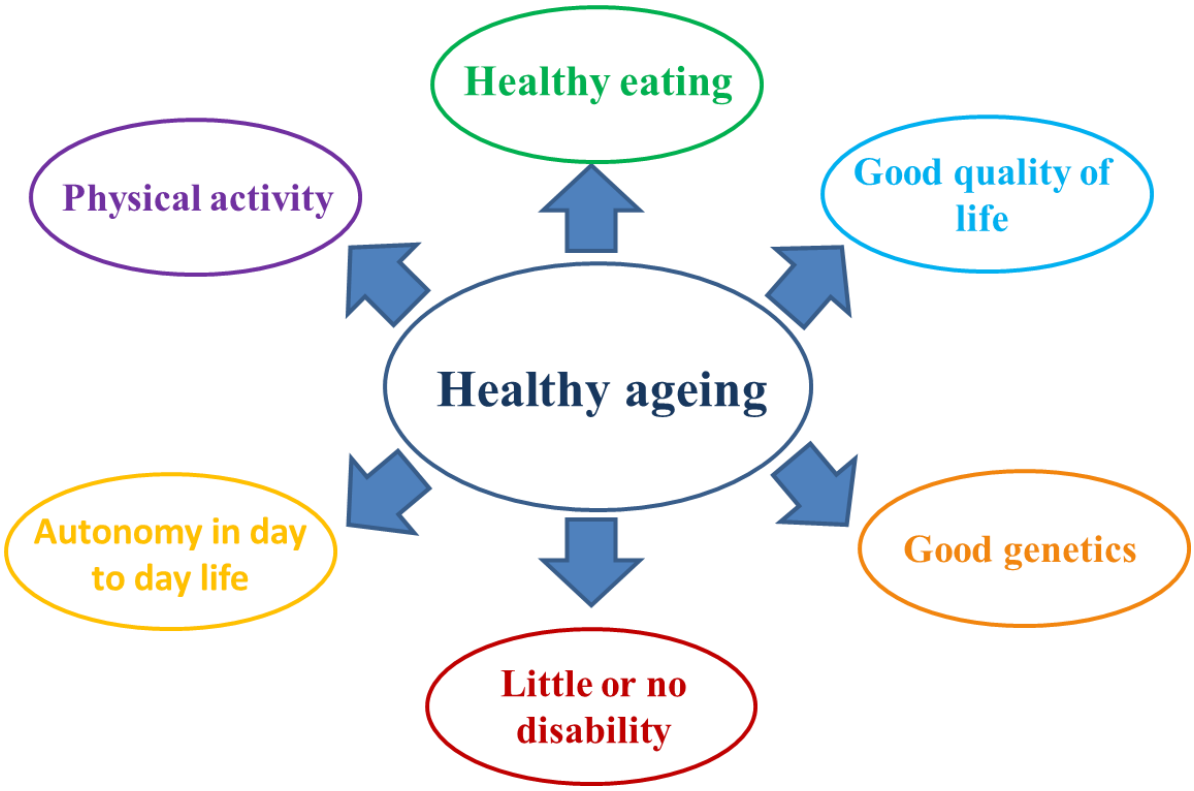

Figure 1. The main characteristics of healthy ageing.

I.I Ageing process and longevity

The major theories regarding ageing, including those involving genes, mitochondria, caloric intake, oxidative stress and so on, are all specific to a particular cause of ageing. These provide useful and important insights in the understanding of age-related physiological changes. However, the ageing process includes several of these mechanisms. So, a global and common view is necessary as the process is still obscure in some of its aspects.

In May 2012, a group of scientists and clinicians met in Athens and drafted a consensus statement to highlight the features of ageing, different ageing processes, longevity and exceptional longevity in human beings. They analysed ageing theories taking into account the evolutionary perspective and proposed treatments suitable to delay the ageing process. According to them, ageing is defined as a physiological and ineluctable process, an intrinsic feature of life. It is characterised by a gradual decrease of the ability to adapt to stress, causing a decline in functional capacity. This event, characterised by a progressive loss of physiological integrity of many interrelated systems, leads to impaired function and increased risk of morbidity and mortality (*Avery et al., 2014*).

On the other side, human longevity is a complex phenotype influenced by environmental factors (socio-economic status of parents, education, epigenetics, medical assistance, and lifestyle) which accounts for 75%, whilst the contribution made by genetics has been estimated to be 25-30% (*Deelen et al., 2013*). Among NSPs genes, the insulin/insulin-like growth factor (IGF)-1 signaling pathway plays a central role in the attainment of longevity. This pathway regulates lifespan in various organisms, including

mammals. Moreover, mutations that reduce the activity of insulin/IGF-1 signaling can extend lifespan in several species of organisms (*Fontana et al., 2010*).

The ageing process, independent of gender, involves organisms at all levels. Some ageing phenotypes such as grey air, hearing loss, presbyopia, short-term memory loss, are not dangerous for old people. Whereas other like changes in cells, tissues and organs (and their progressive reduction of function), increase the vulnerability that leads to death (*Troen, 2003*).

As a definition, there are two ways to become old: without success (unsuccessful ageing, UA) and with success (successful ageing, SA). The first is manifested by people that develop one or more age-related diseases, such as neurodegenerative (Alzheimer's or Parkinson's disease), metabolic (metabolic syndrome and type 2 diabetes mellitus) and cardiovascular diseases (CVDs), and cancer (*Troen, 2003*).

With regards the latter, gerontology distinguished between two groups of non-diseased older persons: usual (non-pathologic but high risk) and successful (low risk and high function). SA includes three main related components, amongst which are: low probability of disease and disease-related

disability, high cognitive and physical functional capacity, and active engagement with life. So, SA is more than absence of disease or the maintenance of functional capacities, because the combination of these components guarantees an active end of life, that represents the essential concept of SA (*Bülow and Söderqvist, 2014*) (**Figure 2**).

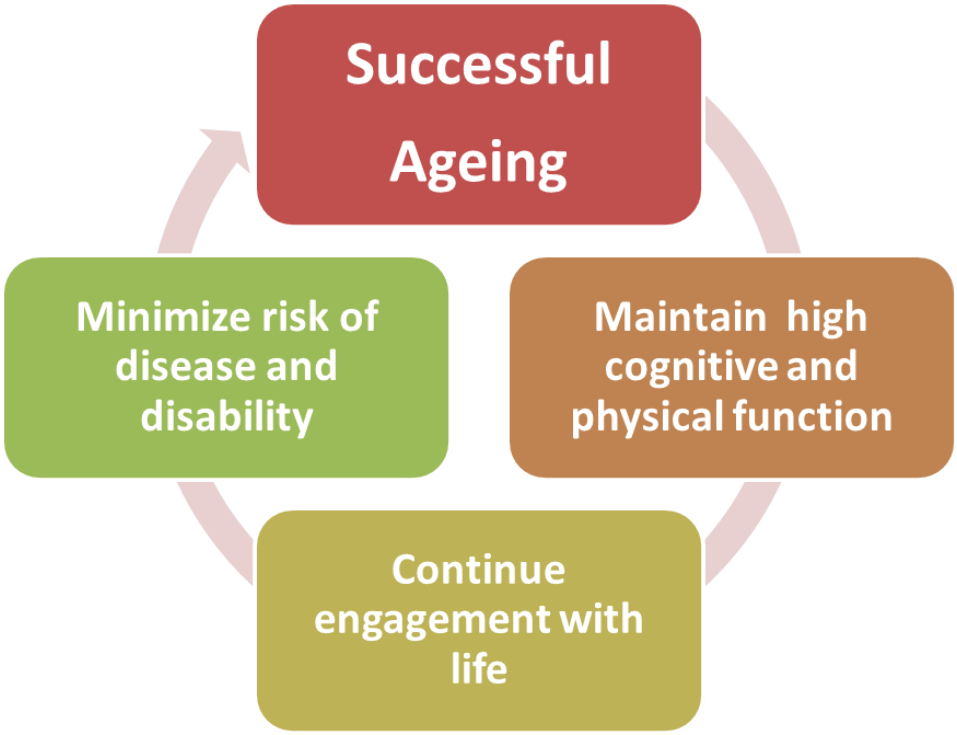

Figure 2. Focus on factors that contribute to SA.

Centenarians, people that live 100 or more years without any significant disease, in good physical and mental condition, represent the best model of SA (*Avery et al., 2014*).

They have a favourable genetic background, essential to live longer, and a good responsiveness to adverse environmental conditions (*Balistreri et al., 2012*). In particular, our group has extensively studied the Sicani Mountains (a small area of Sicily) and the dietary habits of their inhabitants, who showed a high percentage of centenarians. In 2012, it was demonstrated that this area is characterized by a low mortality rate due to cancers and CVDs and the centenarians recruited tended to be physically active and to have a healthy diet, strictly adherent to the Mediterranean dietary pattern (*Vasto et al., 2012a; Vasto et al., 2012b).*

Likely, the centenary's ability to reach the age of 100 (mostly in the westernized countries), in addition to dietary pattern, reflects the improvement of hygienic conditions, the reduced exposure to infection and inflammation, the improvement in general of the quality of life, and the advent of therapeutic and preventive medicine (*Vasto et al., 2012a*).

The strict adherence to healthy diet can also help to reduce the inflammatory status that characterizes ageing, the inflammageing. This process consists in the presence of a chronic low-grade inflammatory status which contributes to the

development of different pathological conditions (*Franceschi et al.*, 2000; *Cevenini et al., 2013*).

In early life, when natural selection is strong, the inflammation should be beneficial because protects the organism from harmful conditions and contributes to healing process. On the contrary, at a later stage of life, this particular inflammatory activity leads to long-term tissue damage and is related to mortality risk for all causes. This phenomenon is mainly attributed to a continuous antigenic stimulation, not foreseen by evolution.

Principally, inflammageing may derive from age-related changes to the immune system, the so-called immunosenescence (*Franceschi and Cossarizza, 1995; Vasto and Caruso, 2004*). In line with the remodeling theory of ageing, in immunosenescence, innate immunity is largely conserved or even up-regulated, while the clonotypic variety deteriorates with age. This reduces the ability to clear novel pathogens and increases T-cell populations, functionally distinct in that the production of pro-inflammatory cytokines is amplified (*Pawelec, 2012; Caruso and Vasto, 2016*). Paradoxically, centenarians show high levels of pro-inflammatory markers, counterbalanced by anti-inflammatories

ones (*Franceschi et al., 2007*). Other causes of inflammageing present themselves through damaged macromolecules and the endogenous host-derived cell debris that, accumulating with age, as a consequence of their increased production and/or inadequate elimination, are sources of chronic damage (*Franceschi et al., 2000*). The amount of senescent cells increases with age and can have deleterious effects on tissues, altering the function of nearby cells. This is due to the acquisition of the senescence-associated secretory phenotype that induces senescent cells to produce pro-inflammatory cytokines (*Tchkonia et al., 2013*). Visceral obesity is known to be associated with a pro-inflammatory status (*Balistreri et al., 2010*). Moreover, the autophagy, a self-degradative process, important for balancing sources of energy in development and in response to nutrient stress, declines with ageing, enhancing the inflammageing process. In particular, defects in mitochondrial uptake and degradation could increase reactive oxygen species (ROS) production and stimulate the inflammatory process (*Salminen et al., 2012*). Finally, harmful metabolites, produced by the human microbiota, likely participate in the ageing process, contributing to

inflammageing and immunosenescence (*Woodmansey, 2007*) (**Figure 3**).

Chronic inflammation negatively impacts on physiological functions, causing all age-related disabilities. At this regard, current available evidence strongly suggests that an anti-inflammatory diet can potentially restrict this condition. Indeed, a healthy nutritional pattern can reduce pro-inflammatory status, offering a unique, nonpharmacological approach in treating obesity, metabolic syndrome, diabetes and other age-related diseases. Moreover, several important bioactive dietary components can exert their effect modulating glucose or insulin levels and inflammatory pathways that can affect metabolic changes (*Santoro et al., 2014*). On the contrary, physical inactivity and a hypocaloric diet lead to an accumulation of visceral fat and to a stimulation of immune-system, causing an infiltration of pro-inflammatory macrophages in adipose tissue (*Balistreri et al., 2010*).

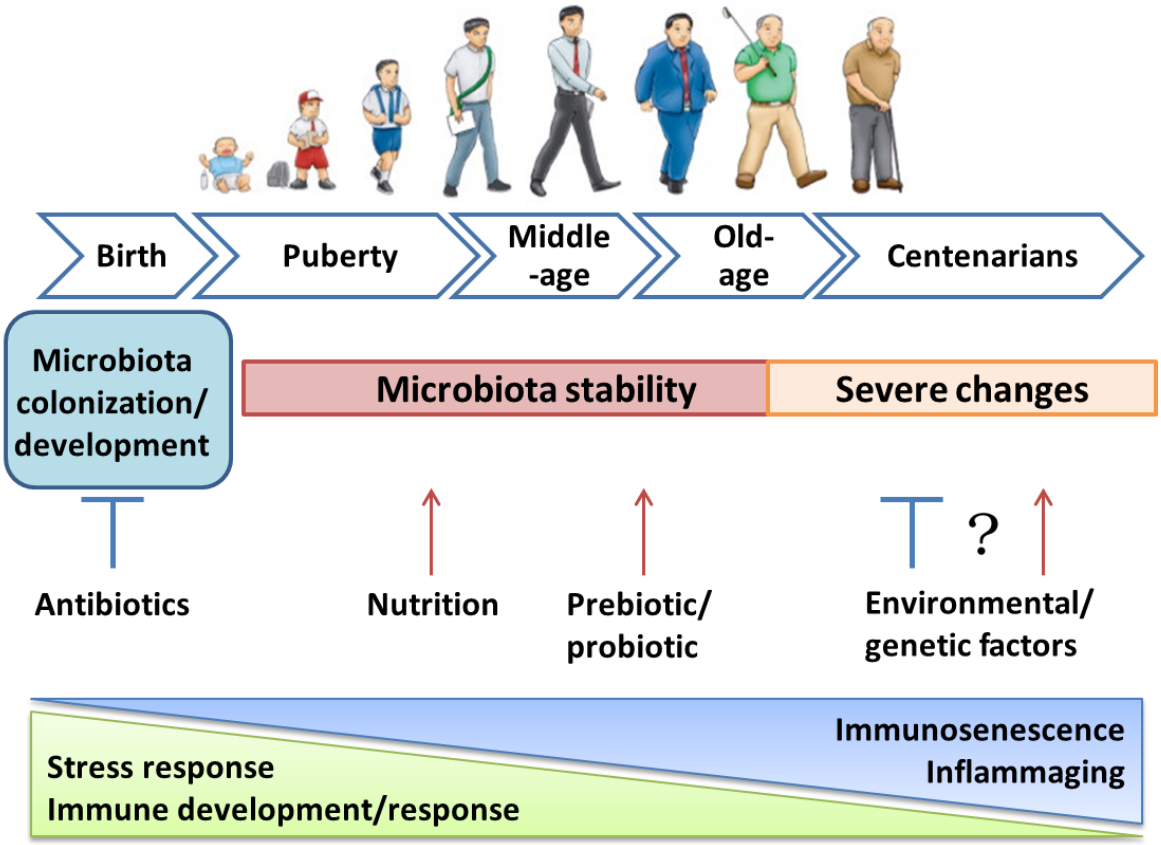

Figure 3. Inflammaging, immunosenescence, and gut microbiota have implications for health and lifespan. Emerging evidence has revealed extensive crosstalk between microbiota, the immune system, and inflammation pathways that influences ageing in humans. This interplay is mediated by various genetic and environmental factors such as nutrition and lifestyle.

I.II E*xcursus* on traditional dietary interventions and their potential impact on human morbidity and lifespan

It is well established that a healthy nutritional pattern plays a central role in preventing diseases and promoting

health. Dietary regimens that can slow or postpone ageing process, promoting long life and healthy old age, continue to raise interest among the general public as well as the scientific and clinical communities.

Among these, several types of DR approaches exist. DR, defines as “Dietary regimen in which specific food groups or micronutrients are reduced or removed from the diet”, was first showed about 80 years ago to extend lifespan in rats (*Mirzaei et al., 2014*). Most of these dietary interventions provide a significant health-span increase also in humans, minimizing adverse effects and increasing longevity (*Lee and Longo, 2016*).

Caloric restriction (CR), the reduction of total calories intake by 20-40% without malnutrition, is the most well-known defined dietary intervention to delay ageing in model organisms. From unicellular yeast to primates, the effects of CR on health-span have been confirmed, suggesting a highly conserved role of some common ageing pathways (*Fontana et al., 2010*; *Most et al., 2016).* The molecular mechanisms that mediate the effect of CR are still being investigated and, in all models, it was seen that reduced nutrient signaling is associated with the downregulation of NSPs, such as insulin and IGF-1,

the amino acids targets of rapamycin, and the glucose signaling Ras-protein kinase A pathway (*Fontana et al., 2008*; *Speakman and Mitchell, 2011*; *Slack et al., 2015*).

The role of CR in human ageing is difficult to ascertain because human lifespan makes long-term investigations and there are no universally accepted biomarkers to measure the rate of human ageing (*Butler et al., 2004*). However, some epidemiologic and short-term human studies support CR-related health benefits (*Fontana et al., 2010*; *Heilbronn et al., 2003*; *Holloszy et al, 2007*; *Lee et al., 2001*; *Walford et al., 2002*). In 2004, a long-term (>30 years) epidemiologic study linked CR to human longevity. This study suggested that maintaining a modestly low energy intake (mean kcal/day 1882) in mid-life involves the lowest late-life mortality risk. Indeed, a weak trend towards lower all-cause mortality was reported in healthy never-smoking Japanese–American men whose caloric intake had been reduced by 15% with respect to the cohort average (*Willcox et al., 2004*). Moreover, CALERIE (Comprehensive Assessment of the Long-termEffects of Reducing Intake of Energy), a controlled randomized study on non-obese individuals, reported that a two year 25% CR is feasible for humans and provides health benefits, such as

reduces inflammatory markers and cardio-metabolic risk factors. However, it was seen that in enrolled subjects CR was associated with reduced bone mineral density (*Ravussin et al., 2015; Redman et al., 2014*). So, considering the results in model organisms, more and long-term studies are required on healthy human populations in order to understand the consequences of CR on ageing and lifespan.

Another investigated dietary approach is fasting. It is an extreme dietary intervention, consisting of a complete lack of food or of a 60% or higher food restriction, that has been practiced as a treatment for many illnesses since ancient times. Only recently have several studies shown the beneficial aspects of fasting in adaptive cellular responses. It reduces oxidative stress damage and inflammation, optimizing energy metabolism and enhancing cellular protection, and results in ketogenesis, promoting potent changes in metabolic pathways (like lipolysis and autophagy) (*Longo et al., 2015*).

The best-characterized form of fasting, evaluated in both rodents and human studies, are intermittent (IF) and prolonged fasting (PF). Intermittent abstinence from food and caloric beverages at least 12 hours (IF) or 48-120 hours (PF), improves health effects and protect from age-related

disabilities. In humans, IF (e.g., consumption of approximately 500kcal/day for 2 days a week) has beneficial effects on insulin, blood glucose levels, C-reactive protein, and blood pressure (*Harvie et al., 2011*). On the other side, in mice, PF cycles, lasting 2 or more days, but separated by a week of normal diet, have emerged as a highly effective strategy to reduce circulating IGF-1 and glucose levels, and, consequently, to down regulate the mammalian target of rapamycin (mTOR) pathway. In humans, IF and PF help to reduce obesity, hypertension, asthma and rheumatoid arthritis (*Lee et al, 2012*; *Longo and Mattson, 2014*).

Nevertheless fasting has the potential to delay ageing and has rare and usually reversible side effects, other diets that mimic fasting were investigated. In 2014, Longo and Mattson, after their studies on Laron syndrome (autosomal recessive disorder characterised by an insensitivity to growth hormone) tuned a plant-based fasting mimicking diet (FMD), which promotes health-span (see **Figure 4** for an overview on FMD results on models). The authors discovered that, in mice, cycles of the FMD lasting 4 days, followed by a standard *ad libitum* diet, determined a decrease of blood glucose and IGF-1 levels, and an increase of ketone bodies compared to a control diet

group. The results also demonstrated that FMD cycles can have profound effects on visceral fats and promote immune system regeneration and rejuvenation (*Cheng et al., 2014; Longo and Mattson, 2014*).

Interesting results have been also demonstrated about the role of FMD in murine models in cancer treatment. The data indicates that FMD cycles combined with chemotherapy can enhance tumor immunogenicity, in part by a heme oxygenase 1 dependent mechanism, which promotes the recruitment of cytotoxic T cells in the tumor bed (*Di Biase et al, 2016*). In addition, still in mice, FMD reduces autoimmunity and multiple sclerosis symptoms and improves cognitive performance and multi-system regeneration (*Brandhorst et al., 2015*; *Choi et al, 2016*).

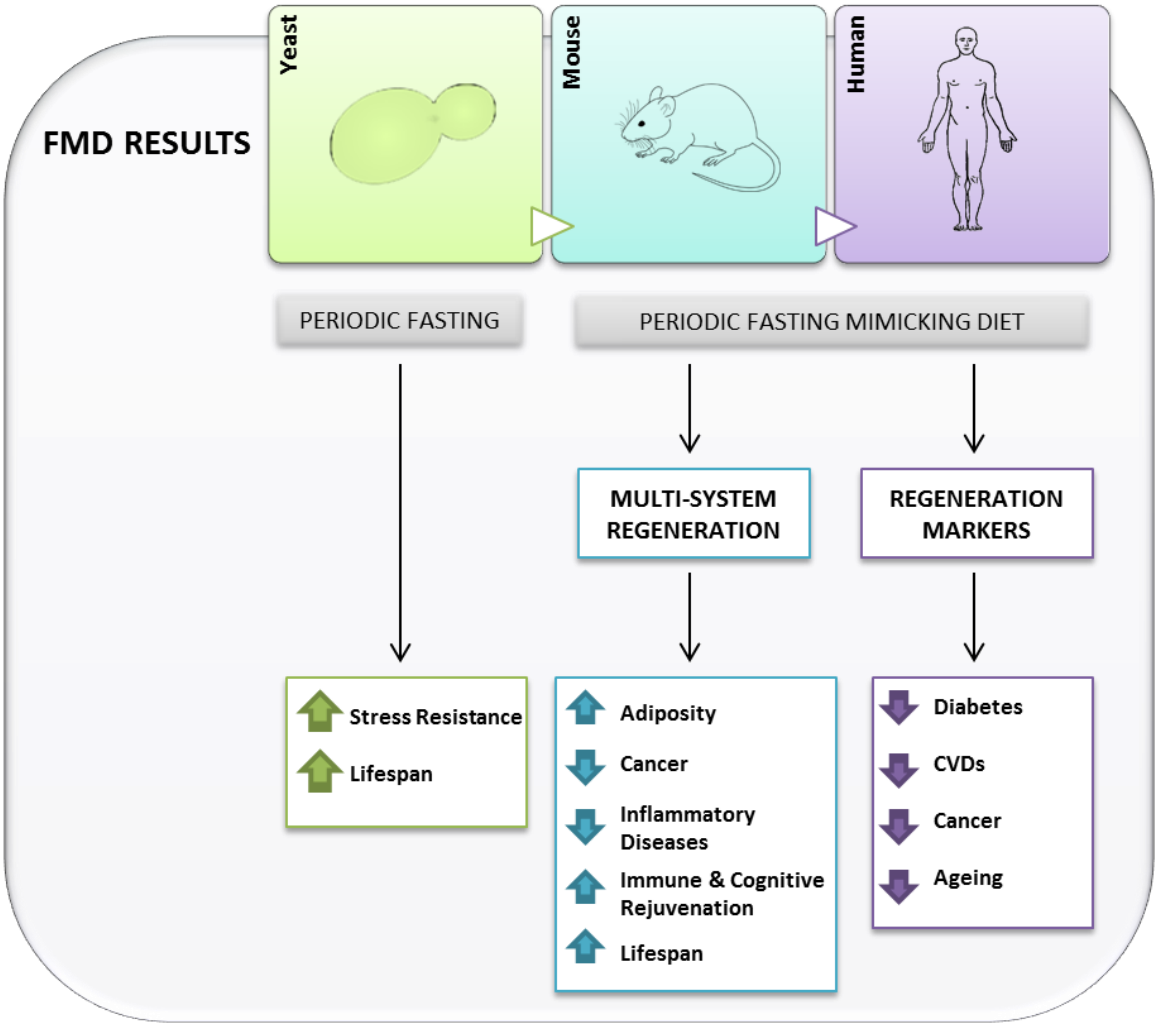

Figure 4. FMD test results. It was demonstrated that a periodic meal program that mimics fasting promotes multi-system regeneration, enhanced cognitive performance, and health span. Yeast models, deprived of food periodically, were shown to have longer life expectancy than yeast fed normally. In mice, a specialized diet for four days a month, improved metabolism and cognitive function, gradual weight loss and muscle rejuvenation, immune system regeneration, and longer average, though not absolute, life expectancy. In human beings, after five FMD days for three months, results showed that the FMD participants experienced an overall improvement of the risk of the main age-relate diseases.

Recently, Longo and colleagues tested the effects of three cycles of low calorie and low protein FMD in a pilot

randomized clinical study using 38 healthy subjects, 19 cases and 19 controls, with a broad range of ages (19-75). The fasting plan was characterised by low carbohydrate and protein intake and contained good fatty acids, and was administered for 5 days per month for each 3 months (3 cycles) (**Table 1**). At the end, the consumer can go back to his normal lifestyle/diet for the remaining 25 days of the month.

	1° day	2° day	3° day	4° day	5° day
Calorie intake	1.090	725	725	725	725
Proteins	10%	9%	9%	9%	9%
Fats	56%	44%	44%	44%	44%
Carbohydrates	34%	47%	47%	47%	47%

Table 1. 5 days of FMD: Calorie and nutrients distribution.

It was seen that FMD normalizes many metabolic health metrics correlated with ageing and health-span. In particular, this diet would seem to reduce the levels of fasting blood glucose, circulating IGF-1, and C-reactive protein, and to increase serum ketone bodies. Moreover, the FMD resulted in a 3% reduction in body weight together with a positive trend of

reduction for fat mass (probably associated with the ketone bodies production), and a positive trend of increase for mesenchymal stem- and progenitor cells, indicating beneficial health effects for regeneration parameters in humans (*Brandhorst et al., 2015*).

In addition, in studying the benefits attributed to PF or IF based strategies as alternatives to CR more deeply, the scientists understood that the restriction of specific macronutrients, without the restriction of calories, is one of the most promising interventions in the promotion of healthy ageing in humans. Among these, the restriction of protein (protein restriction, PR) or the restriction of particular amino acids (amino acid restriction) are the most effective pro-longevity interventions in multiple model organisms (*Mirzaei et al., 2014*; *Grandison et al., 2009*). Indeed, these dietary patterns are sufficient to reduce IGF-1 concentrations and mTOR signalling, more than simple CR (*Fontana et al., 2008*; *Soultoukis and Partridge, 2016*). In *Drosophila melanogaster*, the effect of CR is lost in the presence of essential amino acids (*Grandison et al., 2009*; *Iwasaki et al., 1988*). Especially, methionine and tryptophan, contained more in animal than in plant proteins, seem to have more detrimental effects than other

amino acids. Their reduction, in the diet of various model organisms, extends lifespan and increases stress resistance, with though an unclear molecular mechanism. (*Mirzaei e al., 2014*).

However, few studies were performed on the potential benefits of PR on the human ageing process, and one of the main limitations of these studies is the age stratification of analysed groups. In a case-control study, an association between a low protein consumption, a reduction in IGF-1 levels and cancer in 50-65 years people was demonstrated, but not in the older population, suggesting that a higher protein intake, mostly from vegetables, should be recommended for them (*Levine et al., 2014*).

As regards the plant protein effect, a short-term randomized clinical trial has shown that high plant protein intake was inversely associated with mortality. Plant proteins have not been linked with an increase of IGF-1 levels but have been linked to lower blood pressure, improved insulin sensitivity, reduced low-density lipoprotein (LDL) levels, and lowered the incidence of CVDs. Moreover, it was seen that rats fed by soy proteins have a higher lifespan than those fed by casein (*Song et al., 2016*).

CR and other dietary interventions are not always applicable. As just stated, solid evidence for the positive effect of efficient high-quality diet on human health, rather than a restricted intake, also exists. A close adherence to the Mediterranean Diet (MedDiet) or Okinawan one, that also include healthy lifestyle and low caloric intake, plays a central role in the incidence of age-related disabilities, such as CVDs, diabetes mellitus, and cancer, in a large population sample (*Pallauf et al., 2013*).

Okinawans are the longest-living population in the world, according to reports made by the World Health Organization (WHO) and Japanese Ministry of Health. They seem to have delayed the ageing process and minimized the debilitating diseases that accompany the later years, especially CHDs. Much of the longevity advantage in Okinawa is related to its traditional diet which is low in calories but dense in vitamins, minerals, and phytonutrients. It is characterized by relatively high consumption of unrefined, low glycemic index sugars from vegetables, legumes and fruits, with moderate fish and marine food consumption (*Willcox et al., 2007*). Traditional Okinawan cuisine centers on the sweet potato, green-leafy or yellow-root vegetables, and soy (e.g. miso soup,

tofu or other incarnations of this legume) which accompanied almost every meal. Smaller servings of fish, noodles, or lean meats flavored with herbs, spices, and cooking oil are often associated with the Okinawan dishes (*Willcox et al., 2014*) (see **Figure 5** for Okinawa diet pyramid).

Several studies suggests that other factors play a fundamental role in Okinawans longevity including: high calcium intake from foods and natural drinking water, high flavonoid intake, high vitamin D levels from exposure to sunlight and increased physical activity, especially at older ages (*Suzuki et al., 2001*).

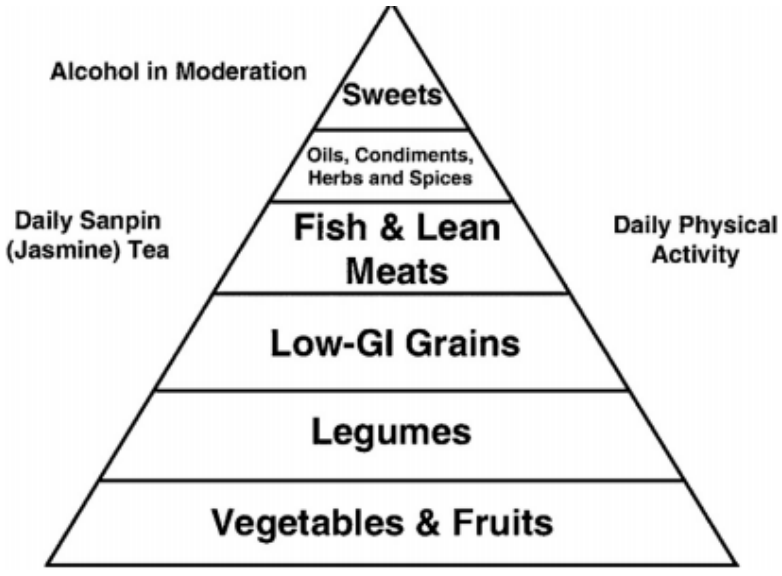

Fig. 1. Traditional Okinawan diet food pyramid. Okinawa diet pyramid.

Figure 5. Traditional Okinawa diet food pyramid (*Willcox et al., 2014*).

I.III Mediterranean diet: The most investigated dietary pattern for achieving successful ageing

Among the many dietary interventions, the MedDiet has received much attention since its beneficial influence on health and longevity has been consistently demonstrated (**Table 2**). Several clinical trials and some meta-analyses showed significant support for the positive effect of a greater adherence to the MedDiet on mortality and morbidity from different causes. So, it has been promoted as a model for healthy eating and contributes to a favorable health status and to a better quality of life (*Martinez-Gonzalez et al., 2009; Sofi et al., 2014*; *Sofi et al., 2008*; *Vasto et al., 2014a*).

Macronutrient	MedDiet	CR	FMD	PR	Western Diet
Protein (animal)	Low	Low/normal	None	Low	High
Protein (plant)	Moderate	Low/normal	Low	Low	Low
Saturated fats	Low	Low/normal	Very low	Normal	High
Unsaturated fats	High	Low/normal	High	Normal	Low
Sugars	Low	Low/normal	Very low	Normal	High
Carbohydrates	High	Low/normal	High	Normal	Low

Table 2. Macronutrient composition of MedDiet compared with other dietary interventions.

The traditional MedDiet, which more than a diet could be defined a lifestyle, is the common dietary pattern adopted by inhabitants of countries within Mediterranean basin where the olive tree, *Olea europaea*, is widely cultivated for the production of table olives and oil. It involves a set of knowledge, rituals, symbols, traditions, and cooking, that contribute to healthy lifestyle, reducing the risk of developing age-related diseases and disabilities, and promoting healthy ageing. Eating together is the foundation of the Mediterranean culture and the hospitality, neighborliness, intercultural dialogue are some of its fundamental aspects. For all these reasons, in 2010, the MedDiet was inscribed on the representative list of intangible cultural heritage of humanity (*UNESCO, 2010*).

MedDiet was first defined by Ancel Keys as being low in saturated fat and high in vegetable oils, observed in Greece and Southern Italy during the 1960s. Keys and his colleagues hypothesized that it can have beneficial effects on a range of diseases, indicating that it meets several important criteria for a healthy diet (*Keys et al., 1965*).

A universally defined Mediterranean pattern does not exist as within the Mediterranean countries there are many

cultural, religious ethnicity, economic development and other factors that contribute to the diversity of dietary patterns (*Trichopoulou 2012*). Therefore, the concept of the 'MedDiet' is more accurate than the concept of a specific diet in particular. However, dietary patterns that prevail in the Mediterranean regions have many common characteristics such as: a high consumption of fruits, vegetables, legumes, and non-refined cereals, including bread; a high ratio of monounsaturated to saturated dietary lipids (mainly olive oil); a moderate consumption of milk and dairy products; a low consumption of meat and meat products, and alcohol. Fish intake is a desirable characteristic of the MedDiet but it has depended on the proximity to the sea. Mediterranean lifestyle is also characterized by daily physical activities, the consumption of particular seasonal foods, and a high intake of β-carotene, group B, C, and E vitamins, folic acid, polyphenols and other phytochemicals (*Trichopoulou A, 2004*).

This diet also consists of a low intake of animal proteins, it is hypocaloric, characterized by low amount of cholesterol and sugars, and a high content of fibers. Although it is quite difficult to define the type of fibers in MedDiet, this term is generally used to indicate the plant residues that are resistant to

digestion by enzymes from the intestinal lumen. The fibers are distinguished as either soluble or insoluble: soluble fibers acting mainly in the first part of the digestive tract (stomach and small intestine), with the insoluble fibers being more active in the terminal part of the digestive tract (large intestine).

According to the American Diabetes Association, an adequate consumption of dietary fiber from a variety of plant foods, preserves populations from chronic disease. Indeed, the intake of dietary fiber has beneficial effects with regards to risk factors for developing several chronic diseases. Epidemiologic studies have shown that a suggested consumption of 14 g dietary fiber per 1,000 kcal, or 25 g for adult women and 38 g for adult men, gives protection against cardiovascular disease (*Slavin et al., 2008*). Moreover, it was highlighted that the fibers can promote the colonic health, by stimulating the growth of beneficial gut microflora (thus acting as prebiotics) (*Del Chierico et al., 2014*).

Despite most people in the Mediterranean areas only partially adhering to MedDiet guidelines, there is a sufficient number of people, especially in some regions, where the presence of centenarians is high. This determines the link

between the MedDiet and health (*Mirzaei at al., 2016*; *Vasto et al., 2014b*).

Nowadays, the data that support the role of the MedDiet on health derive from biochemical, clinical and epidemiological research that are great in number and availability, and all confirm its association with reduced mortality in old age and with success both in improving cognitive function and reducing risk of chronic illnesses like cancer, metabolic syndrome, neurodegenerative pathologies, and, mostly, CVDs (*Buckland et al, 2011*; *Chrysohoou et al., 2013*). This is true not only in Mediterranean basin, but also in northern Europe, Australia, Spain, the USA (*Kiefte-de Jong et al., 2014*), and in other countries were the MedDiet is widespread.

For example, *Sofi et al*., in a meta-analysis, evaluated the correlation between a strict adherence to MedDiet and the low mortality for CVDs, CHDs and strokes in Mediterranean basin. They showed that an increase in MedDiet was inversely associated with death by CVDs (*Sofi et al., 2014*). In that regard, it has been established that moderate drinking of red wine, a typical Mediterranean beverage, reduces the risk of CVDs and CHDs, probably by increasing levels of serum high

density lipoprotein (HDL) cholesterol (*Rimm et al., 1996*). Moreover, more important is the intake of monounsaturated lipid, mostly from olive oil and olives that directly contributes to increased HDL cholesterol levels (*Al-Khudairy et al., 2015*). In addition, benefits attributed to MedDiet have emerged comparing the Mediterranean pattern with low-carbohydrate and high protein diets, which are often associated with metabolic syndrome and cancer (*Fung et al., 2010*). After all, a positive correlation between red and processed meats and risk of cancer or other age-related diseases such as diabetes, is already known (*Wolk, 2016*), and, conversely, there is strong evidence that the consumption of vegetables and fruits reduces the risk of most forms of cancer (*Schwingshackl et al., 2016*).

Furthermore, consuming a diet with a high percentage of calories coming from added sugars has been linked to increased risk of obesity, type 2 diabetes mellitus, dyslipidemia and other risk factors for CVDs. In particular, prolonged consumption of added sugar is linked to an increase of LDL and to a decrease of HDL, and an increase of blood inflammatory markers (*Malik et al., 2010a and b*).

In summary, as CR evidence on model organisms suggest, more beneficial effects of Mediterranean dietary

pattern are closely related to low glycaemic index (GI) and low animal protein intake. Both of them are able to act directly on NSPs. In particular, low glycaemic and protein intake could reduce blood glucose levels and therefore insulin release, downregulating insulin/IGF-1 and mTOR pathways, and, consequently, activating some transcription factors (TFs) implicated in the homeostatic gene transcription. It promotes longevity, decreases inflammatory status and oxidative stress. These beneficial effects could also be attributable to the presence of specific Mediterranean nutraceuticals and functional foods (see **I.III.II**).

So, it is not certain that the MedDiet can fully explain the good health of Mediterranean people, but it appears unquestionable that it contributes to it preservation.

I.III.I Focus on Mediterranean pyramid: History and evolution

Since 1995, the MedDiet has been popularized using a graphical representation of the pyramid, which has been updated over the years. The first MedDiet pyramid (MDP), created using the current nutrition research of that time, was based on the dietary traditions of Crete, Greece and southern

Italy in the early 1960s, where the rates of chronic diseases among these populations were the lowest in the world.

This first pyramid was represented using the world famous pyramid representation that graphically highlights the food groups to be consumed daily, weekly or less frequently. It included regular physical activity and moderate consumption of wine, and was low in saturated fatty acids. The diet was characterized by abundant plant foods and derivatives (fruits, vegetables, breads, pasta, rice, polenta, potatoes, beans, nuts, and seeds), olive oil as the principal source of fats, and dairy products (principally cheese and yogurt). A daily intake of these foods was recommended. Fish and poultry were consumed in low to moderate amounts, zero to four eggs weekly, red meat, and sweets a few times for month (**Figure 6**).

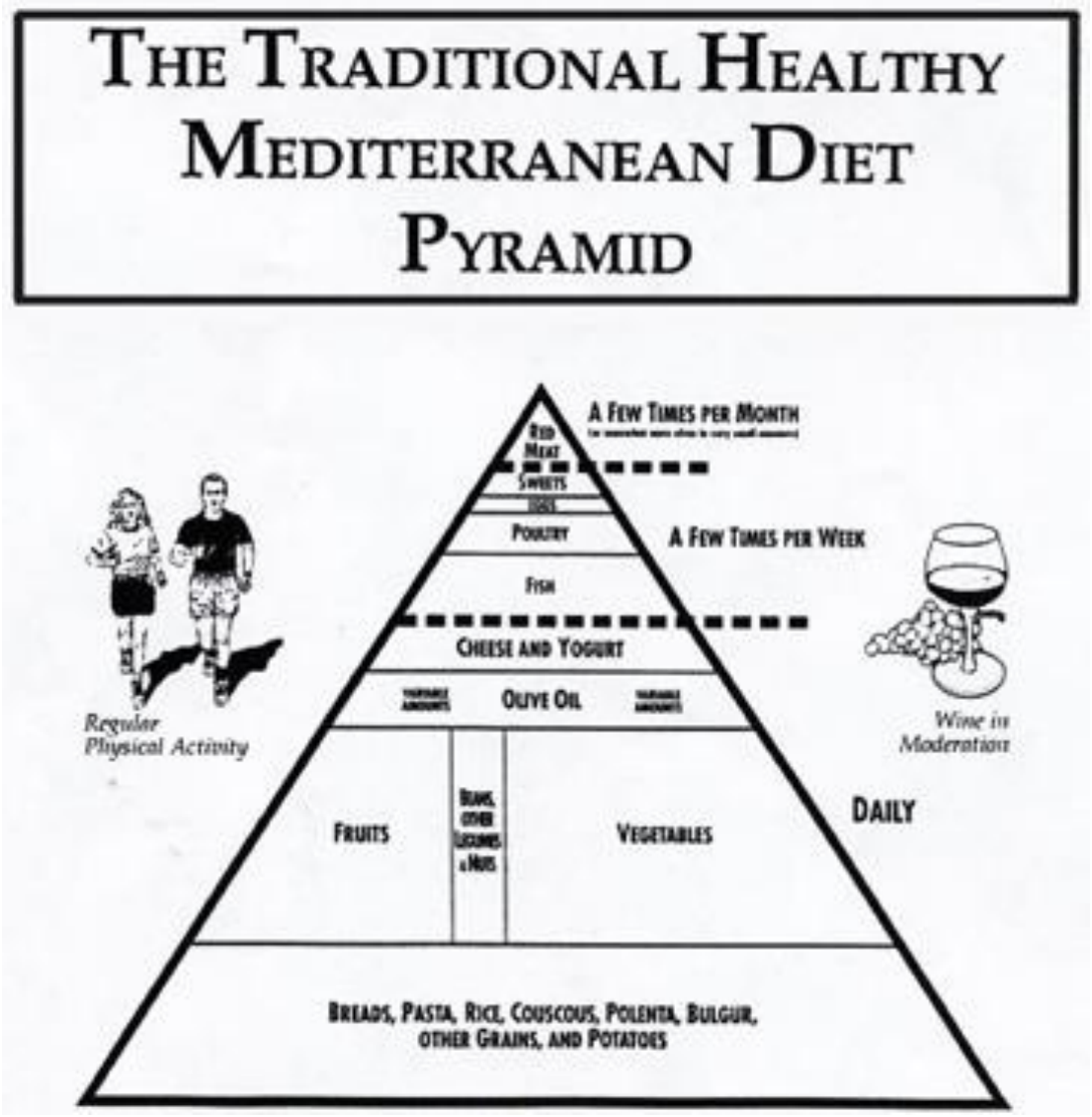

Figure 6. The traditional healthy MedDiet pyramid in 1960.

In 2008, after the 15th Anniversary Mediterranean Diet Conference, a consensus position on a new revised MDP was promoted. The new feature of the MDP update was based on the definitive scientific evidence for the healthfulness of Mediterranean eating and drinking patterns. Three principal

modifications are incorporated in this updated MDP: all plant foods (fruits, vegetables, grains, mostly whole, nuts, legumes, seeds, olives and olive oil, herbs and spices) are placed in a single group at the base of the pyramid, indicating that they should be the basis of most meals; the frequency of consumption of fish and seafood is increased to at least two times per week, indicating their multiple contributions to brain and reproductive organ health; herbs and spices are added to the pyramid to reflect increased evidence of their health-promoting characteristics and to contribute to the national identities of various Mediterranean cuisines (**Figure 7**).

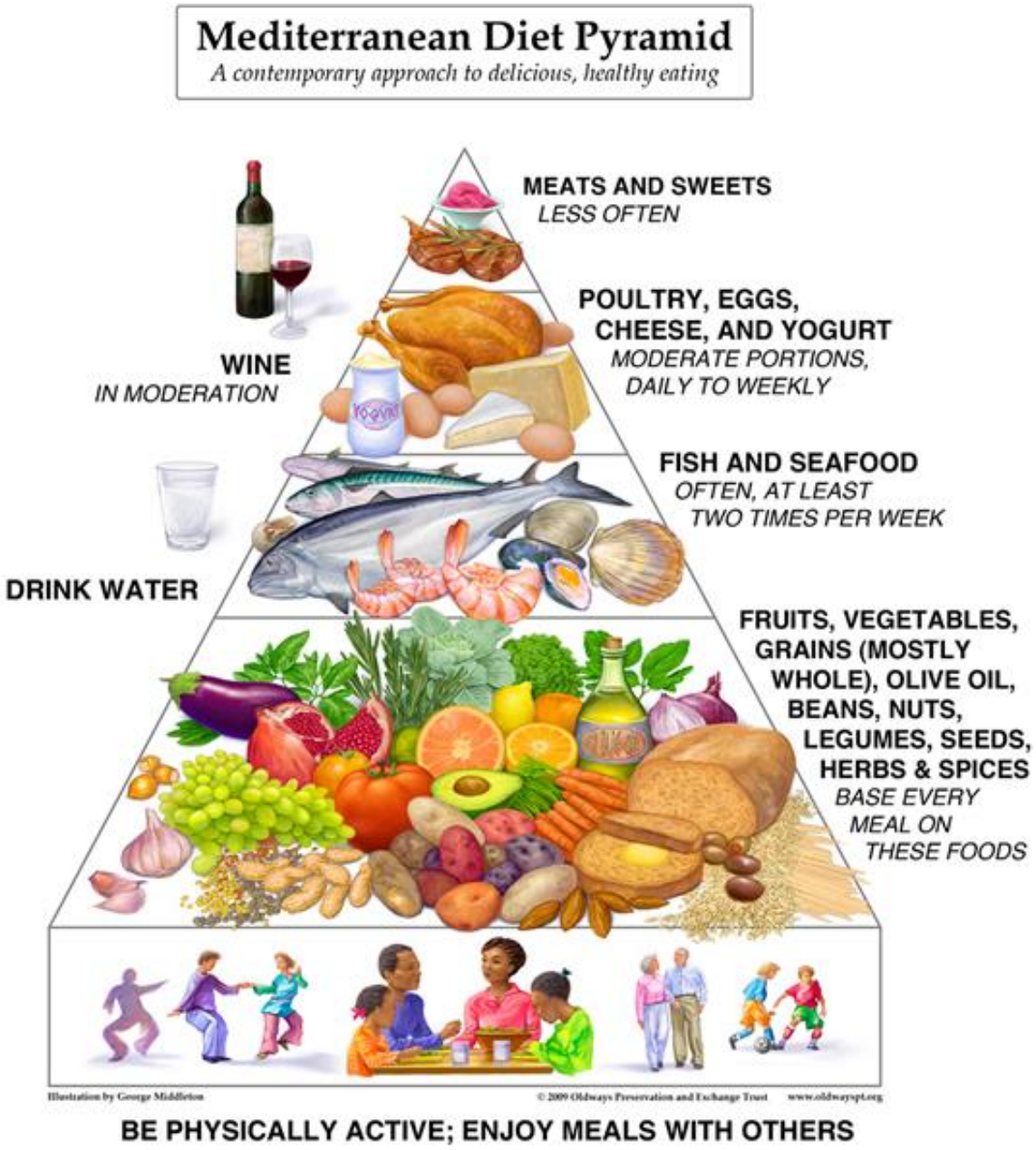

Figure 7. The MedDiet pyramid in 2008.

In 2010, after the recognition of the MedDiet as intangible cultural heritage of humanity by UNESCO, scientists present a third model of pyramid to evidence the

contribution of the strict adherence to this dietary pattern to a healthy life. The new revised MDP arises from the internal dialogues among scientific experts after the conference about 'The Mediterranean Diet today, a model of sustainable diet' in Parma (Italy). This pyramid results from the latest research in the field of nutrition and health, scientific evidence around the healthiness of the Mediterranean dietary pattern, and its role in the prevention of many chronic diseases by large epidemiological studies. The final design of the MDP today provides key elements for the selection of foods, both quantitative and qualitative, indicating the relative proportions and consumption frequency. Plant-origin foods, that represented the heart of the MedDiet, are situated at the base of the pyramid, and are responsible for the prevention of many chronic diseases and for weight control. Moreover, at the base foods that provide a high energy intake are also present, and at the upper levels, foods to be eaten in moderate amounts such as those of animal origin and/or rich in sugars and fats. The pyramid establishes daily, weekly and occasional dietary guidelines in order to ensure a constant healthy and balanced diet. Among the recommendations regarding the proportion and frequency of food consumption, in the 2010 MDP the

cultural and lifestyle elements deemed necessary to acquire all the benefits from the MedDiet, and to preserve its cultural heritage, were incorporated (*Bach-Faig et al., 2011*) (**Figure 8**).

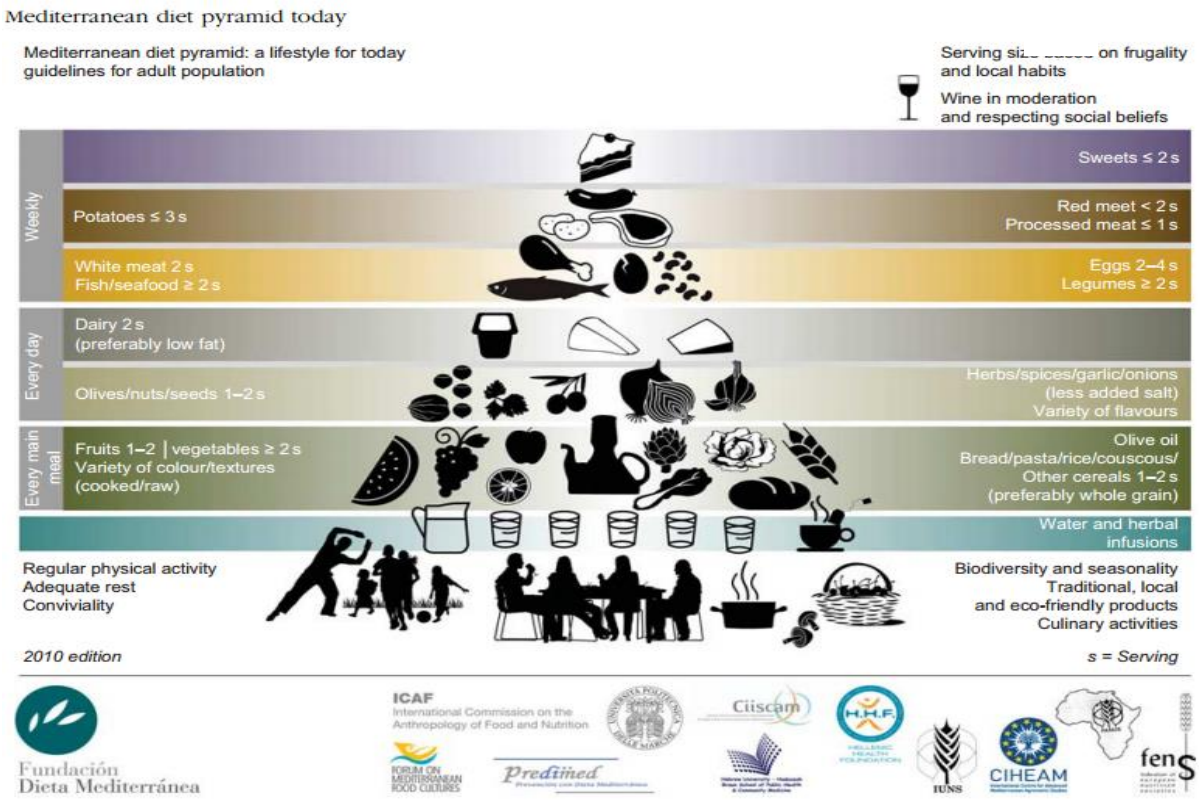

Figure 8. The MedDiet pyramid in 2010: A lifestyle for today (*Bach-Faig et al., 2011*).

I.III.II Mediterranean nutraceuticals and functional foods: The role on healthy ageing

In addition to the effects on NSPs, likely attributable to low GI and low animal protein intake, the MedDiet is renowned to be rich in plant foods and derivatives (fruits, vegetables, nuts, seeds, wine, olives and olives oil) which play a fundamental role in reducing some risk factors in age-related diseases (*Vasto et al., 2014b*). Recent clinical trials and epidemiological studies indicate that the putative beneficial effects of a high intake of fruits and vegetable on the risk of diseases of ageing may be due to antioxidant compounds. However, other non-antioxidant phytochemicals contained in plant foods may be equally important.

In recent years, chemistry research into natural products has focused on the identification of biologically active secondary metabolites produced by plants, traditionally used as medicinal remedies. The most well-known of these being vitamins and minerals. Nevertheless, plants contain other secondary metabolites which are defined as “nutraceuticals” and which are at the center of important scientific studies.

The term nutraceutical is a *portmanteau* word, a combination of “nutrition” and “pharmaceutical”. It was coined

by DeFelice in 1989 and refers to “Naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products, and have health promoting, disease preventing, or medicinal properties” (*DeFelice, 2002*; *Gupta et al., 2010*). Nutraceuticals have, mainly, antioxidant and anti-inflammatory effects that confer the MedDiet its anti-ageing features. They act on the inflammatory status and on the prevention of oxidative reactions, resulting in a significant reduction of all risk factors for CVDs. Furthermore, they result in the improvement of cognitive function and in the decrease of chronic degenerative disorders such as cancers, Alzheimer’s, Parkinson’s, and autoimmune diseases (*Aruoma, 2003*; *Santoro et al., 2014*; *Vasto et al., 2014a; Visioli and Galli., 2001*).

Examples of Mediterranean nutraceuticals are: polyphenols, terpenoids, flavonoids, alkaloids, sterols, pigments, vitamins, and unsaturated fatty acids. All the foods that contain these compounds can be considered as “functional foods”. Although a universal definition of them does not exist, the Functional Food Center defined them as “Natural or processed foods that contain known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven and documented health

benefit for the prevention, management, or treatment of chronic disease" (*Martirosyan and Singh, 2015*).

Among nutraceuticals, epidemiological studies have attributed many of positive effects on wellness to the presence of polyphenols, compounds having antioxidant activity. From a chemical point of view, they are classified as phenolic acids, flavonoids, stilbenes, and lignans, and are widely present in fruits and vegetables, including those found in the Mediterranean. In plants, they are involved in the defence against pathogen attacks or stress induced by chemical and physical damage. Flavonoids, such as flavones, anthocyanins, flavans, isoflavones, and so on, contained in fruits, vegetable and legumes, act as protection against diseases such as cancer and CVDs in humans. They also possess anti-allergic, anti-inflammatory, antiviral, anti-proliferative, and anti-carcinogenic properties (*Prior and Cao, 2000*). Catechins and procyanidins, contained in the seeds and skins of grapes, are the major constituents of red wine, along with resveratrol (*Greca and Zarrelli, 2012*). They are also found in chocolate, other fruits, and vegetables. Their importance has been extensively demonstrated in literature and, in particular, their role as an inhibitor of cancer cells, in the reduction of

atherosclerotic plaques, and in the prevention of CVDs (increasing HDL and lowering LDL blood levels, and inhibiting LDL oxidation) is particularly interesting (*Ruidavets al., 200*0). Anthocyanins, another group of flavonoids, like quercetin, are present in fruits with purple skins such as red grapes, blueberries and strawberries, and are correlated with a minor risk of type 2 diabetes mellitus (*Muraki et al., 2013*; *Wedick et al., 2012*). Anthocyanins too have interesting antioxidant and anti-inflammatory power which is explicated in cardio-protective effects, principally through the action on LDL blood levels. Additionally, resveratrol, an antioxidant non flavonoid polyphenol (mainly found in red grapes and red wine), lycopene and other carotenoids (pigments found in tomatoes and in other yellow and orange fruits and vegetables), and folic acid, in general, can alleviate the inflammation in the cardiovascular degenerative processes, such as atherosclerosis, improving nitric oxide (NO) release and the endothelial function (*Accardi et al., 2016a*).

These natural substances, consumed as fresh foods or fresh extracts, are always more active than other preparations because of the absence of deterioration due to the processing, and have a greater intestinal absorbability.

Most likely, some polyphenols carry out their antioxidant effects by reducing the expression of some TFs, like kappa-light-chain-enhancer of activated B cells (NF-κB), implicated in the inflammatory process, and by enhancing the activation of proteins, like sirtuins, that also by inhibiting NF-κB, can reduce the cellular stress response (*Vasto et al., 2014a*). Moreover, new data have suggested that a small amount of polyphenols is able to promote other cellular stress response pathways, for example the nuclear factor erythroid 2-related factor 2 (Nrf2) that activates genes encoding antioxidant enzymes.

Another class of nutraceuticals with anti-inflammatory effects are mono/poly unsaturated fatty acids (MUFA and PUFA), like oleic acid, omega-3 and omega-6. Numerous studies have demonstrated that the ingestion of fats rich in omega-6 and omega-3 fatty acids suppress *de novo* hepatic fatty acid biosynthesis, triglyceride production and triglyceride secretion, while they enhance hepatic and skeletal muscle fatty acid oxidation (*Clarke et al., 2002*). In addition, regulating lipid metabolism, unsaturated fatty acids may improve insulin sensitivity and increase non-oxidative glucose metabolism. Moreover, they are important for governing the rate of

conversion of dietary carbohydrate to fatty acids and triglycerides. It can be speculated that a diet rich in olive oil, abundant in MUFA, combined with a balance of dietary fat derived from marine and vegetable lipids, rich in PUFA, may reduce the risks associated with excessive tissue triglyceride accumulation, which in turn may delay the onset of obesity and type 2 diabetes mellitus (*Vasto et al., 2014a*).

So, nutraceutics is the science that deals with the study of foods, or parts of those foods, that have particular beneficial, preventative and therapeutic effects on human health. Often the word nutraceutical is compared to the adjective “functional” even if a functional food is not necessarily said to be a nutraceutical. A food is therefore considered to be functional when it is used as for its ability to prevent or treat specific diseases.

Functional foods are: yoghurt, some fruits and vegetables rich in antioxidants, such as broccoli, olives, *Opuntia Ficus Indica* (OFI), olive oil, nuts, and foods enriched with nutraceutical extracts or with fibers. Several careful studies on Mediterranean populations, whose traditional diets consist largely in these foods, show low incidences of certain

chronic diseases and long life expectancies (*Ortega, 2006*) (see **Figure 9** for the main Mediterranean functional foods).

Growing evidence suggests that extra virgin olive oil (EVOO), the main source of fat for Mediterranean people, must play a key role in explaining the health benefits of the MedDiet.

It is the juice of olives obtained by a mechanical procedure and consumed after washing, filtration, and decantation, or centrifugation, and under controlled temperatures that will not harm the oil (*Virruso et al., 2014*). It is used for cooking and as a dressing and its nutraceutical properties derive from the presence of a complex mixture of about 200 bioactive compounds. EVOO composition depends on many factors which include geographical origin, weather and irrigation, ripening and processing after harvesting. The main constituents of olive oil are triglycerides, the so-called “saponifable fraction” (98–99%). The three main fatty acids in the triglyceride fraction are: a MUFA (oleic acid), a saturated fatty acid (palmitic acid) and a PUFA (linoleic acid). The remaining “unsaponifable fraction” (1-2%) contains about 230 bioactive molecules. These include: lipophilic phenols (tocopherols) whose levels fall as olives mature; sterols; color pigments,

mainly chlorophylls and carotenoids (the most important being beta-carotene); alcohols; waxes, aldehydes, esters, and ketones; and phenolic compounds (hydrophilic phenols). Their quantity is strongly influenced by the variety and the geographical origin of the olives. For example, Greek *Koroneiki* olives have a very high level of polyphenols, while the polyphenol content of the Spanish *Arbequina* variety is low, and the polyphenol content of Sicilian *Nocellara* is medium-high (*Aiello et al., 2015*).

Evidence shows that EVOO decreases the pro-inflammatory environment, induced by oxidized LDL, both in experimental models and in humans, also reducing the C-reactive protein, a powerful marker of inflammation, and the interleukin (IL)-6 levels, as shown in dietary interventions (*Schwingshackl et al, 2015*). With its high content of MUFAs and polyphenols, EVOO might exert beneficial effects on the development and progression of age-related diseases. Oleic acid is the main MUFA and is claimed to decrease LDL oxidation. It has also been suggested that MUFA can decrease all-cause (11%) and cardiovascular mortality (12%), cardiovascular events (9%), as well as strokes (17%) (*Schwingshackl et al, 2015*). Moreover, oleic acid suppresses

cytotoxic function of natural killer cells and consequently the anti-inflammatory effect (*Yaqoob et al., 1994*). In general, unsaturated fatty acids improve endothelial function, decreasing intercellular adhesion molecule-1 production by endothelial cells, and reducing leukocyte adhesion (*Bemelmans et al., 2002*). In fact, both *in vitro* and *in vivo* studies, demonstrated the endothelium-protective properties of these molecules (*Accardi et al., 2016a*; *Schwingshackl et al, 2015*; *Virruso et al., 2014*). Different trials showed that polyphenol EVOO intake has been associated with low mortality rates caused by CHDs (*Saita et al., 2015*). Indeed, these compounds are able to bind LDL, increasing resistance to oxidation and acting as radical scavengers (*Gimeno et al., 2007*). Moreover, it was seen that the consumption of polyphenol-rich olive oil can decrease blood pressure and improve endothelial function in young women with high-normal blood pressure (*Moreno-Luna et al, 2012*). Generally, polyphenols inhibit NF-κB pathway that lead to the expression of pro-inflammatory genes. In particular, hydroxytyrosol and oleocanthal have ibuprofen-like activity, inhibiting cyclooxygenases 1 and 2, responsible for prostaglandin production (*Virruso et al., 2014*).

Several health claims for EVOO and its derivatives have been assessed but only one was authorized in Europe. It relates to the impact of olive phenolic compounds on the protection of blood lipids from oxidative stress: “A daily intake of 20 g of olive oil, which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein and tyrosol) provides the expected beneficial effects” (*European Community, 2007*).

Moreover, our pilot study demonstrated that daily consumption of green table olives *Nocellara del Belice* is likely linked to a decrease in IL-6 and malondialdehyde (MDA) levels (*Accardi et al., 2016b*). MDA is the main product of the PUFA peroxidation and is an important index of oxidative stress (*Czerska et al., 2015*). It is worth noting that our study highlighted a reduction of fat mass with an increase of muscle mass of subjects recruited for the nutritional intervention. The possible explanation could be linked to the capacity of conjugated linoleic acid (CLA) to reduce the body fat levels, strictly linked with the production of adipokines (pro-inflammatory cytokines) (*Lehnen et al., 2015*). CLA is present both in EVOO and table olives, and can also be produced during their digestion. In experimental models, acting as

signalling mediators, CLA inhibits lipogenesis, increases fat oxidation, and reduces adipocytes size (*Fazzari et al., 2014*; *Wang et al., 2004*) (see **VI.**).

Furthermore, pasta is another traditional food of MedDiet. The possibility to enrich it with fibers, that lower the GI, make pasta a product eligible to be a processed functional food (*Bjork et al., 2000*; *Tudorica et al., 2002*). In particular, it is known that (1-3, 1-4)-β-D-glucans, commonly referred to as β-D-glucans, are one of the most abundant forms of polysaccharides found in the cell wall of yeasts, fungi, some bacteria, algae and cereals. They are soluble fibers, contained from 1% in wheat grains, to 3-7% in oats, and 5-11% in barley (*Skendi et al., 2003*). These soluble dietary fibers have hypocholesterolemic effects, which improve glycemic control (*Jenkins et al., 2000*). The effect on cholesterol depends on the ability to form a viscous layer on the surface of the small intestine. The higher viscosity reduces the intestinal absorption of cholesterol and the reabsorption of bile acids. The inhibition of the reabsorption of bile acids can increase the synthesis of bile acids from endogenous cholesterol, and reduces the circulation of cholesterol LDL by about 8 % (*Othman et al., 2011*). A minimum dose of 3 g/day of β-glucans has been

suggested to reduce the levels of cholesterol in the blood and decrease the risk of cardiovascular diseases. In a recent pilot study, our group demonstrated that after 30 days of intake of pasta with 6% of β-glucans, there was a significant decrease of LDL-cholesterol, IL-6 and advanced glycation end-products (AGEs), confirming the capacity of β-glucans intake to lower oxidative stress and inflammatory status (*Barera et al., 2016*). Successively, we showed that the same pasta was able to increase endogenous levels of some gut hormones such as glucagon like peptide (GLP)-1 and peptide YY (PYY), implicated in the modulation of glucose tolerance and food intake. This intervention also showed a promising effect on gut microbiota changes (see **VII.**).

Finally, the increased prevalence of obesity and metabolic syndrome has led the proliferation of various supplements on market with varying claims of effectiveness. Some of these supplements consist of OFI extracts, a typical and widespread plant of the Mediterranean basin (*Onakpoya et al., 2015*).

OFI is a domesticated cactus plant and its fruits were often investigated for their antioxidant and anti-inflammatory properties. Its leaves, the cladodes, contain mainly pectins,

carotenotenoids, polysaccharides, polyphenols, vitamins, polyunsaturated fatty and amino acids, and their extracts, rich in fibers, have health positive effects. The identified natural OFI compounds and derivatives were shown to have biologically relevant activities including hypoglycemic, antimicrobial and neuroprotective properties (*El-Mostafa et al., 2014*). In humans, the consumption of OFI seems to confer protection against peptic ulcers and to improve platelet function, and it represents a natural remedy for gastroesophageal reflux. Randomized and nonrandomized human studies have shown that OFI could attenuate postprandial hyperglycemia by stimulating glucose uptake in the peripheral tissues, and reduce total cholesterol (TC), LDL levels, and triacylglycerols in patients with dyslipidemias, though unknown mechanisms (*Alecci et al., 2016*; *Onakpoya et al., 2015*).

These promising evidence has stimulated our group to study the actual properties and effects of a pasta made with OFI cladode extracts on hematochemical, anti-inflammatory, antioxidant and anthropometric parameters, to test its potential hypoglycemic, hypocholesterolemic and weight loss properties and its possible modulator action on gut microbiota (see **VIII.**).

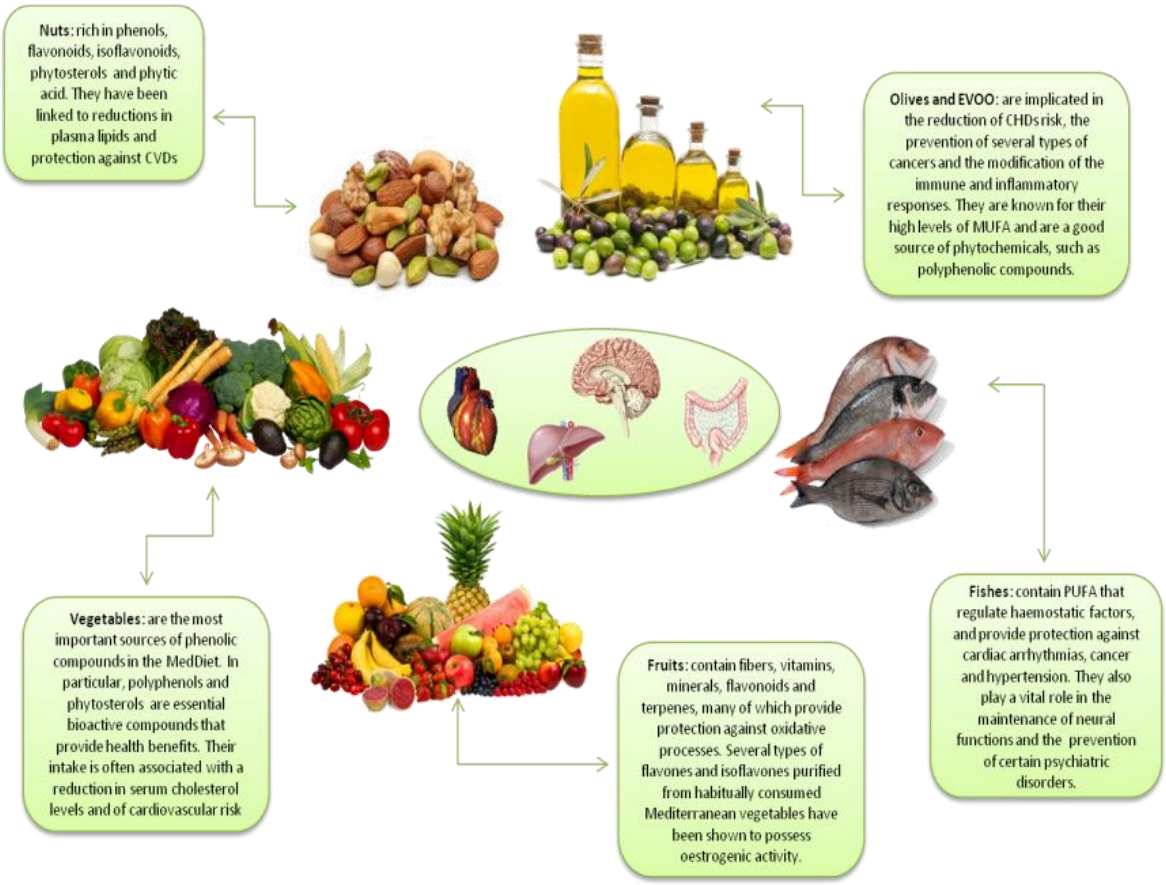

Figure 9. The main Mediterranean functional foods and their health benefits. Among the functional food present in the MedDiet are fruits and vegetables, nuts, olives and olive oil, as the prevalent fats for dressing, fish, as the main source of protein and polyunsaturated/saturated fats. The health benefits coming from the food of the Mediterranean diet resides on the presence of bioactive compounds such as polyphenols, carotenoids, phytosterols, fibers, MUFA and PUFA, displaying antioxidant activity, protection from cancer and hypertension, and improvement of the intestinal functions.

II. Aim of the thesis: Different approaches to investigate the role of functional foods on human healthy ageing

The interventions to slow or postpone ageing, favoring the health-span, represent the new challenges in ageing investigation (*Longo et al., 2015*). Some mechanisms that delay and prevent the onset of ageing disabilities have been identified and lifestyle, nutrition and pharmacological approaches have emerged.

In particular, as it is clear from this thesis work, eating habits represents the most concrete way to act on the ageing process, extending our productive lives, without frailty.

Thus, the aim of my PhD research was to investigate the role of Mediterranean functional foods on health, focusing the attention on their anti-inflammatory and antioxidant effects, and on the possible action on microbiota alterations.

At first, we explored the literature to summarize the existing data regarding the beneficial properties of the MedDiet on healthy ageing and on the molecular pathways that are the basis of these effects. So, we have identified some foods with nutraceutical capacities, and we have tested them through *in vivo* nutritional interventions. In particular, we analyzed three

Mediterranean functional foods: table green olives *Nocellara del Belice*, a pasta enriched with 6% of β-glucans, and a pasta with 3% of OFI cladode extracts added. After, we decided to test some EVOO extracts on peripheral blood mononuclear cells (PBMCs, *ex vivo* interventions), obtained from volunteer recruitment.

II.I *In vivo* studies: the nutritional intervention

A nutritional intervention is a clinical study involving human volunteers, also called participants, who have conscientiously decided to lend themselves to the service of science. During the intervention, the participants are subjected to a specific diet or to require to consume specific food, or part of this, with the aim to analyze its action on some health parameters. To date, these trials represent the best model to study the consequence of nutrition in humans.

We performed three nutritional interventions to evaluate the effect of table green olives, pasta with β-glucans, and pasta with OFI cladode extracts, on clinical and biological parameters of engaged individuals. In the first case, we recruited 25 randomized volunteers, both men and women. The subjects included were: healthy, aged between 18 and 65, and

Caucasian. The trial consisted in the consumption of 12 olives/day for 30 days. The green olives belonged to the variety *Nocellara del Belice*, and were processed in salt solution without any chemical additives. At baseline (T0) and at the end of the intervention (T1), hematochemical tests, oxidative stress and cytokine analyses, anthropometric measurements, and microbiological analyses on feces, were carried out (see **VI.**)

The second nutritional intervention consisted of the consumption of 100 g of pasta supplemented with β-glucans, 4 times a week, for 30 days. We enrolled 10 overweight participants, selected between young volunteers. Before and after 30 days of pasta intake, hematochemical analysis have been performed, and some gut hormones, linked to the food intake and glucose tolerance, have been measured. Stool specimens have been studied for the study of microbiota alterations after the trial (see **VII.**).

During the third study, we recruited 41 randomized volunteers with metabolic syndrome, both men and women, with an average age of 47.5. The subjects had to consume 500 g/week of pasta with *Opuntia*, for 30 days. At T0, and after 30 days (T1), anthropometric measurements, hematochemical, oxidative stress and cytokines analyses were performed.

Further molecular analyses, using qPCR assays, were conducted on total faecal DNA, to measure the variation of its amount. (see **VIII.**). See **Figure 10** for the flow-chart of the three studies.

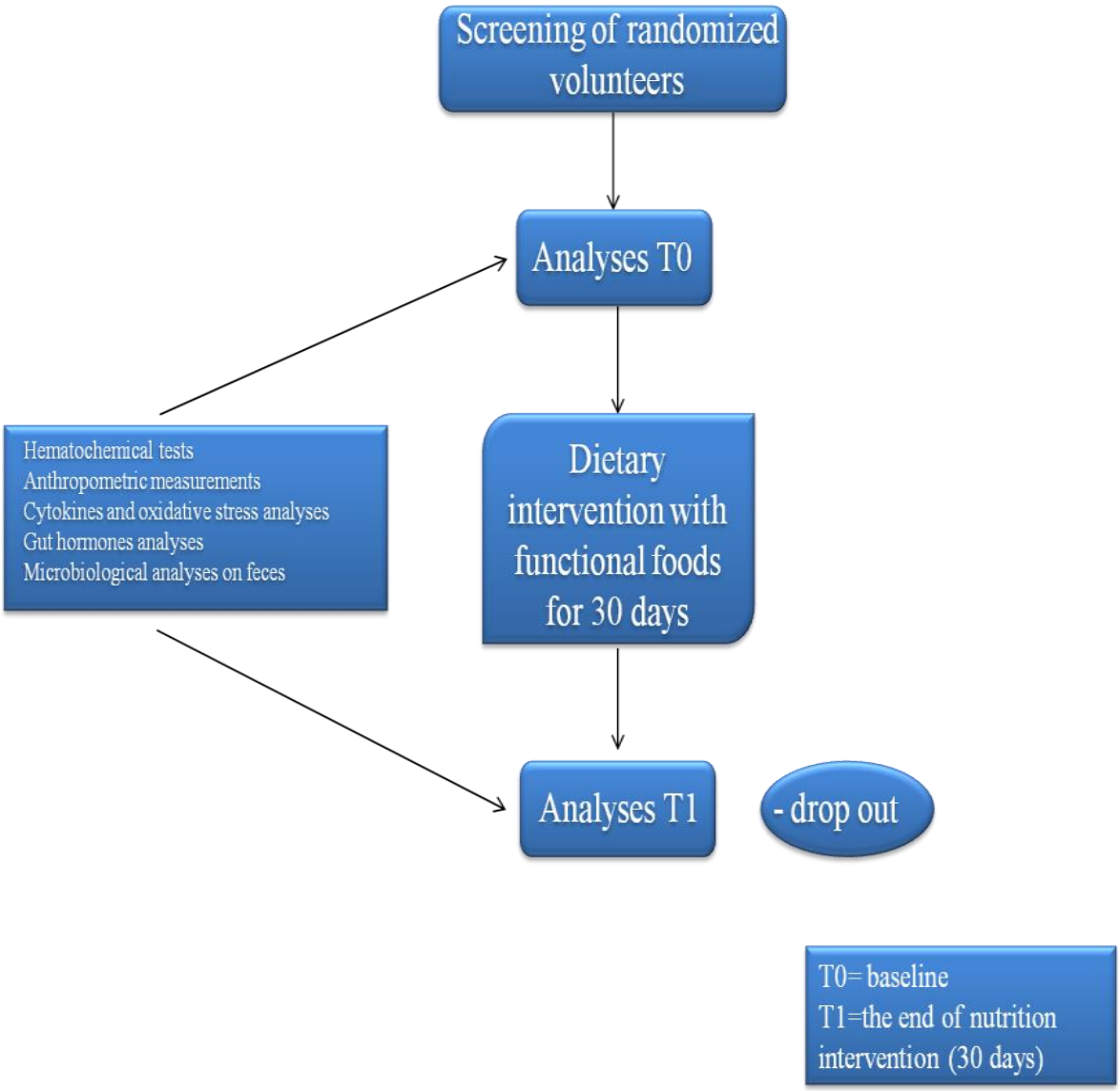

Figure 10. Flow-chart of the nutritional interventions.

II.II *Ex vivo* studies: Cell culture of peripheral blood mononuclear cells

During the second year of my PhD research activity, I spent three months at the *Instituto Maimónides de Investigación Biomédica de Córdoba* (IMIBIC), in Spain, for an Erasmus plus traineeship. In this period, I learned how to set up conveniently treated cell culture of PBMCs.

So, in collaboration with University of *Magna Graecia* of Catanzaro, our group decided to test the antioxidant and anti-inflammatory activities of oleuropein, hydroxytyrosol and their paracetylated derivates on PBMCs, using an *ex vivo* cellular screening system. The cells were extracted from 14 whole blood samples of volunteers subjects (age 23-65), cultured and incubated with the above mentioned extracts. See **IX**. for the experimental protocol.

III. What olive oil for healthy ageing?

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Editorial

What olive oil for healthy ageing?

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The olive tree originated in Asia Minor around 6000 years ago and then spread to all the Mediterranean basin. Olive oil is extracted from the pulp of its fruits [1–3]. Many studies show that the nutritional pattern of the so-called Mediterranean Diet is associated with a lower incidence of age-related diseases related to inflammation and oxidative stress, such as cardiovascular disease, Parkinson's disease, Alzheimer's disease and cancer [4–7]. It is now clear that olive oil, as a main source of fat, must play a key role in explaining the health benefits of the Mediterranean Diet.

So what is in olive oil? It is a complex mixture of over 200 compounds. The composition depends on many factors which include geographical origin, weather and irrigation, ripening and processing after harvesting. Thus all olive oils are not all the same. The main constituents of olive oil are triglycerides, the so-called the saponifable fraction (98–99%). The three main fatty acids in the triglyceride fraction are a monounsaturated fatty acid (oleic acid), a saturated fatty acid (palmitic acid) and a polyunsaturated fatty acid (linoleic acid) [1–3]. The remaining unsaponifable fraction (1–2%) contains about 230 components. These include: (i) lipophilic phenols (tocopherols) whose levels fall as olives mature; (ii) sterols the main sterol being beta-sitosterol; (iii) colour pigments, mainly chlorophylls and carotenoids (the most important is beta-carotene); (iv) alcohols; (v) waxes, aldehydes, esters, ketones; and (vi) phenolic compounds (hydrophilic phenols). The phenolic fraction of olive oil are polyphenols of which there are 7 different subfamilies: anthocyanins, flavonoids, flavones, phenolic acids, phenolic alcohols, acids and secoiridoids. Their amount is strongly influenced by the variety and the geographical origin of the olives. Greek Koroneiki olives have a very high level of polyphenols, while the polyphenol content of the Spanish Arbequina variety is low and the polyphenol content of Sicilian Nocellara is medium-high. In addition oil produced from green olives contains more polyphenols than that obtained from more mature fruit. Furthermore heating, method of extraction, or long processing times and inappropriate storage and packaging can result in polyphenol loss.

This variation in composition has led to an EU directive 2568/91 classifying olive oil (OO) according to quality and purity criteria: (i) virgin olive oil (extra virgin olive oil -EVOO-, virgin olive oil -VOO- and lampante olive oil -LOO-), (ii) refined olive oil -ROO- and (iii) olive oil -OO- (4,5). EVOO (free acidity, expressed as oleic acid, not more than 0.8%) is obtained by mechanical processing, or "cold pressing", that preserves polyphenols. VOO (free acidity, expressed as oleic acid, not more than 0.2%) is also obtained by processes that include washing olives with water, milling, cold pressing and centrifugation. VOO maintains relatively high amounts of unsaponifiable compounds and a large amount of phenolic fractions or tocopherols, but during refining, the majority of polyphenols are subsequently lost [5]. LOO is the virgin olive oil which has a free acidity, expressed as oleic acid, more than 2%. However it is not suitable as food because of its high acidity, poor flavour and unpleasant smell unless it is refined. It is a poor quality oil. ROO (free acidity, expressed as oleic acid, not more than 0.3%) is obtained from virgin olive oils by refining methods such as filtering with charcoal or other chemical or physical filters. This leads to a reduction in polyphenols [5]. OO is a mix of refined and virgin oil (other than lampante olive oil; free acidity, expressed as oleic acid, not more than 0.1%). The term indicates an OO obtained by blending refined olive oil and virgin olive oil other than lampante olive oil.

But, what olive oil is recommended for healthy ageing? As discussed below, the positive effects mostly depend on polyphenols, and EVOO should represent the best choice for healthy ageing. Many studies now support this idea.

Oleic acid represents the topmost monounsaturated fatty acid (MUFA) provided in the diet (~90% of all MUFA) and is the main monounsaturated fatty acid of olive oil. In South-Europe it is mostly provided by olive oil, whereas in the other Western countries it is provided by meat and meat products, added fats, and dairy products [8]. Oleic acid is claimed to increase the resistance of low-density lipoprotein (LDL) to oxidation and, consequently, reduces the risk of atherosclerosis. It has also been suggested that MUFA offers protection from age-related cognitive decline and might be responsible for the hypotensive effects of OO [4,6–8]. However the source of MUFA seems to be important as shown by a recent meta-analysis. It found an overall risk reduction of all-cause mortality (11%), cardiovascular mortality (12%), cardiovascular events (9%), and stroke (17%) when comparing the top versus bottom third of MUFA, olive oil, oleic acid, and MUFA: saturated fatty acid ratio. MUFA of mixed animal and vegetable sources per se did not yield any significant effects on these outcome parameters. However, only olive oil seems to be associated with reduced risk. When focusing on virgin olive oil consumption, the inverse correlation between olive oil and CVD risk found in the meta-analysis is consistent with the fact that olive

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0378-5122/

oil is not just a source of MUFA but also of other biologically active components [8].

OO also contains phenolic compounds (hydroxytyrosol, oleuropein) which have antioxidant, anti-inflammatory and antimicrobial properties. [4,9]. These are thought to reduce atherosclerotic plaque formation and to be neuroprotective [10–12]. There is a European Food Safety Authority (EFSA) approved health claim on olive oil polyphenols (Commission Regulation (EU) 432/2012): 'Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil, containing at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil [13].'

Identification of the best olive oil for healthy aging remains a complex issue dependent not only on the polyphenol content but also on the amount consumed. Extra virgin oil with the highest polyphenol content seems to offer the most benefits.

Contributors

All authors contributed to the writing of the manuscript and have seen and approved the final version.

Competing interest

The authors declare no conflict of interest.

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Anna Aiello [a]
Giovanni Dara Guccione [b]
Giulia Accardi [a]
Calogero Caruso [a,*]
[a] *Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università di Palermo, Italy*
[b] *Istituto Nazionale di Economia Agraria, Italy*

[*] Corresponding author at: Sezione di Patologia generale, Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi dell'Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy.
Tel.: +39 0916555911.
E-mail address: calogero.caruso@unipa.it
(C. Caruso)

IV. Mediterranean nutraceutical foods: Strategy to improve vascular ageing

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Original article

Mediterranean nutraceutical foods: Strategy to improve vascular ageing

Giulia Accardi, Anna Aiello, Caterina Maria Gambino, Claudia Virruso, Calogero Caruso, Giuseppina Candore *

Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134 Palermo, Italy

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ABSTRACT

Ageing is characterized by a decline in all systemic functions. A greater susceptibility to apoptosis and senescence may contribute to proliferative and functional impairment of endothelial progenitor cells. They play an important role in neo-angiogenesis and endothelial repair. Vascular ageing is associated with changes in the structure and functions of vessels' wall. There are many possible causes of this damage. For sure, inflammation and oxidative stress play a fundamental role in the pathogenesis of endothelial dysfunction, commonly attributed to a reduced availability of nitric oxide. Inflammageing, the chronic low-grade inflammation that characterizes elderly people, aggravates vascular pathology and provokes atherosclerosis, the major cardiovascular disease. Nutraceutical and molecular biology represent new insights in this field. In fact, the first could represent a possible treatment in the prevention or delay of vascular ageing; the second could offer new possible targets for potential therapeutic interventions. In this review, we pay attention on the causes of vascular ageing and on the effects of nutraceuticals on it.

1. Introduction

As quoted in Lim and Townsend, (2009), *"Longevity is a vascular question, which has been well expressed in the axiom that man is only as old as his arteries"* (Sir William Osler, 1891).

Many experimental evidences have shown that advancing age is the major risk factor for the development of vascular endothelial dysfunction that, in turn, increases the risk of developing cardiovascular diseases (CVDs), the main cause of death in Western countries (Ras et al., 2013).

In the last decades, vascular endothelium has conquered importance because of extraordinary versatility of its functions. It plays a crucial role in homeostasis by the regulation of vascular tone, blood cell trafficking, immune response, and physiological vascular structure.

Blood vessels are composed of three layers: an intimal monolayer of endothelial cells (ECs), medial vascular smooth muscle and the adventitia or tunica externa. Vascular ECs synthesize and release a wide array of biologically active molecules which act in autocrine or paracrine manner to modulate arterial structure and vasodilatory, thrombolytic and vasoprotective functions. The mediators released from them in response to biochemical and mechanical stimuli induce an alteration of endothelium-dependent vasodilation, defined as endothelial dysfunction. This condition reflects a disrupted balance between endothelial injury and repair mechanisms and is correlated with several disorders present in ageing, such as CVDs, renal dysfunction, Alzheimer's disease or retinopathy (Coleman et al., 2008; Csiszar et al., 2007; Price et al., 2004). Recent works focused on endothelial progenitor cells (EPCs) as an important contributor to endogenous vascular repair, participating in new vessel formation and endothelial regeneration (Balistreri et al., 2015; Kim et al., 2010).

EPCs are a subset of mononuclear cells derived from hemangioblast cells in the bone marrow that have the ability to differentiate into mature ECs. Since their discovery, about a decade ago, a plethora of evidences has supported their existence, origins, and contribution in new blood vessel formation. Circulating EPCs are recruited to endothelial tissues, suffering from hypoxia, and attend to both blood vessel formation and repair, in affected tissues (Yoder, 2012). They can be identified by cellular morphology, including specific cell surface proteins, and by examining the expression of genes encoding surface markers (Balistreri et al., 2015). In the bone marrow or immediately after their migration into the systemic circulation, early EPCs are positive for CD133,

* Corresponding author. Fax: +39 0916555933.
E-mail addresses: giuliabio@gmail.com (G. Accardi), anna.aiello2903@gmail.com (A. Aiello), cmgambino@libero.it (C.M. Gambino), claudiavirruso@hotmail.it (C. Virruso), calogero.caruso@unipa.it (C. Caruso), giuseppina.candore@unipa.it (G. Candore).

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CD34, and vascular endothelial growth factor (VEGF) receptor-2, whereas mature circulating EPCs show a high expression of CD34, VEGF receptor-2, VE-cadherin, and von Willebrand factor. These data suggest that the loss of CD133 may explain the transformation of circulating EPCs into mature endothelial-like cells. However, it is unclear when they lose CD133 (Harraz et al., 2001).

Clinically, vascular ECs are involved in all cardinal signs of inflammation, such as vasodilatation and increased endothelial permeability.

Nowadays, there is a substantial interest in the potential protective effect of lifestyle in vascular damage. The adherence to Mediterranean diet (MD) reduces oxidative stress and acts positively on inflammatory state (Santoro et al., 2014). Moreover, it improves the regenerative capacity of the endothelium, increasing the number of EPCs (Marin et al., 2011). These effects are mediated by nutraceuticals, present in many Mediterranean foods. The World Health Organization defines them as "naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products, and have health promoting, disease preventing, or medicinal properties". The term, coming from the conjunction between nutrition and pharmaceutics, was coined in 1989 by Stephen De Felice.

In this review, we have focused on the causes of vascular ageing and how Mediterranean nutraceutical foods may improve endothelial damage that, ultimately, represents a balance between the magnitude of injury and the capacity for repair. The criteria used to select the mentioned studies depended on the interests and the relevance of the papers with the studies conducted by the authors. Principally, we focused on human data.

2. Role of nitric oxide in vascular endothelium

Inflammation and oxidative stress play a fundamental role in the pathogenesis of endothelial dysfunction, which is commonly attributed to a reduced availability of nitric oxide (NO).

NO is essential for a functional endothelium. In ECs, it regulates the degree of contraction of vascular smooth muscle cells, platelet aggregation, leukocyte adhesion, and endothelial junctional permeability. These functions are mediated by the stimulation of soluble guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP). cGMP activates the cGMP-dependent protein kinase (PKG), which mediates most of NO physiological effects. The NO/cGMP/PKG signaling pathway is dysregulated in several inflammatory diseases, such as Diabetes Mellitus (DM) (Huynh et al., 2014). A new role for the NO/cGMP/PKG pathway has been suggested in vascular endothelium. It was observed that NO inhibits endothelial gene expression of transforming growth factor (TGF)-β, which conducts a major role in the vascular response to injury. Mice deficient in TGF-β die in utero because of vascular defects. Conversely, blood vessels that overexpress TGF-β develop neointimal formation (Saura et al., 2005).

Some researchers have suggested that the reduction of NO bioavailability associated with endothelial dysfunction may increase TGF-β responses, contributing to the pathogenesis of some vascular diseases. NO is produced by three isoforms of NO synthase (NOS), endothelial (eNOS), neuronal, and inducible (iNOS). In endothelial cells, the latter is under control of transcription factors, which are activated by inflammatory cytokines. Moreover, it is capable of increasing overall NO production, far beyond the levels produced by eNOS. All three NOS isozymes have regulatory functions in the cardiovascular system. A common feature of aged vessels is the reduced bioavailability of NO and the increased production of reactive oxygen species (ROS), which lead to endothelium dysfunction (Morris and Billiar, 1994). Furthermore, it was observed that diminished NO bioavailability reduces the mobilization of EPCs, impairing endogenous vascular repair mechanisms (Aicher et al., 2003).

3. Inflammageing: the role in endothelial dysfunction

In last century, prevalence of CVD is significantly increased in the older population. A possible link between CVD and ageing process is represented by chronic low-grade inflammation that characterizes elderly people, termed "inflammageing" by Franceschi et al. (2000). It is known that systemic inflammation linked to inflammageing aggravates the vascular pathology and provokes atherosclerosis (Bonetti et al., 2003; Incalcaterra et al., 2013).

Inflammageing is an important risk factor for both morbidity and mortality in the elderly. It appears to be involved in the pathogenesis of all age-related diseases, such as atherosclerosis, CVDs, type 2 diabetes, metabolic syndrome, sarcopenia, osteoporosis, cognitive decline and frailty, giving rise to the so-called "diseasome of inflammageing" (Franceschi and Campisi, 2014).

An increased expression of genes involved in inflammation and immune responses, higher level of pro-inflammatory cytokines in serum, as well as the activation of Nuclear Factor kappa B (NF-κB) transcription factor signaling, are characteristic markers of the pro-inflammatory phenotype in aged mammals (Wu et al., 2015).

The most important role in this pro-inflammatory state seems to be played by chronic antigenic stress. However, the trigger of the age-related inflammatory diseases is the interaction between chronic antigenic stress, genetic background and environmental factors. In addition, other important stressors are ROS and additional products of oxidative metabolism. As known, ROS hyperproduction and accumulation induce tissue damage, acting on important cellular components, such as lipid membranes, enzymatic and structural proteins and nucleic acids. Consequently to inflammageing and increased ROS production, older persons display a global reduction in the capability to cope to stressors. Moreover, they show a concomitant increase in pro-inflammatory markers and acute phase proteins. The gene expression of these inflammatory markers is modulated by NF-κB, which is activated by the Inhibitor of NF-κB Kinase/NF-κB inducing Kinase and Mitogen-activated Protein Kinase pathways. NF-κB appears to be the major intracellular signaling peptide, mediating the increased expression of endothelial adhesion molecules. In addition, it was observed a marked activation of inflammatory pathways, especially NF-κB, into the vascular wall of DM subjects suggesting its role in the development of endothelial dysfunction (Huynh et al., 2014).

As above discussed, vascular endothelium plays a key role in regulating function and health of arteries. Several researches suggested that with progressive ageing ECs and smooth muscle cells undergo to changes leading arterial stiffness. Arterial endothelial dysfunction refers to functional alterations in the normal endothelial phenotype of arteries that may contribute to the development of atherosclerosis and other vascular disorders (Widlansky et al., 2003). In particular, during ageing inflammatory responses, ECs are phenotypically converted into an activated state that can lead to vasoconstriction, coagulation, and inflammation.

Recent studies have clearly indicated that the increased ROS production can induce the activation of inflammasomes, in particular Nod like receptor 3 (NLRP3). It is an intracellular multiprotein complex that recognizes pathogen-associated molecular patterns and activates caspase 1, promoting the release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 in macrophages. These cytokines provoke a low-grade inflammation in several tissues and, in that way, accelerate ageing process by inhibiting autophagy. Interestingly, NF-κB signaling is a crucial inducer of NLRP3 expression (Salminen et al., 2012).

Autophagy is an ancient housekeeping mechanism, which controls the turnover of cellular organelles and proteins. Accumulating evidences have clearly indicated that autophagy can suppress inflammatory reactions. Ageing is associated with a decline in autophagy, which impairs cellular housekeeping. This alteration leads to protein aggregation and accumulation of dysfunctional mitochondria, which provoke ROS production and oxidative stress.

Genetic studies showed that disturbed interplay between autophagy and the inflammasomes could be a link between inflammageing and vascular pathologies. It was demonstrated that the loss of autophagy proteins, such as Atg16L1, potentiates the production of IL-1β (Saitoh et al., 2008; Wu et al., 2015).

4. Senescence and vascular ageing

The process of cellular senescence can be an important additional contributor to inflammageing, since senescent cells acquire a phenotype named senescence-associated secretory phenotype (SASP). It is characterized by the enhanced secretion of many inflammatory modulators that modify the tissue microenvironment and alter the function of nearby cells. Interestingly, this phenotype was observed not only in cells of immune system, such as macrophages, but also in fibroblast and ECs (Coleman et al., 2013).

The age-related changes of immune system functions are complex phenomena incompletely understood. According to the remodeling theory of ageing, immunosenescence is the result of a remodeling of immune system cellular pool. This complex scenario is characterized by a deterioration of adaptive immunity, while innate immunity is largely conserved or even up-regulated with age (Candore et al., 2010; Franceschi et al., 2000). Immunosenescence of the innate immune system is primarily characterized by reduced cellular production and capability of phagocytosis. A number of reports have provided experimental evidences of the altered function of neutrophils and macrophages in mice and humans. Franceschi et al. (2000) supposed that inflammageing is the result of chronic activation of macrophages termed "macroph-aging". Aged macrophages show an impaired respiratory burst and reactive nitrogen intermediate production that lead to damage of endothelium.

Several authors demonstrated that the acquisition of SASP reduces endothelial regenerative properties, which, in turn, contribute to the development of pro-inflammatory pathological conditions (Freund et al., 2010).

The cellular senescence also implies the impairment of the pool of stem cells, so of the EPCs. This event leads to the loss of reparative capacity and vascular homeostasis, determining the onset of CVDs and atherosclerosis. In fact, EPCs may contribute to the maintenance of the endothelium by replacing injured mature ECs. The EPC number has been shown to be reduced in patients with CDVs, DM, and multiple coronary risk factors (Imanishi et al., 2008).

Rehman et al. (2003) showed that circulating EPCs promote vascular repair or angiogenesis, activating resident endothelial cells by releasing paracrine factors, such as VEGF or hepatocyte growth factor, rather than by supplying of new endothelial cells.

Recently, it was demonstrated a paracrine action mediated by EC-derived microparticles (EMPs). EMPs are small membrane fragments released from activated or apoptotic ECs after injury. They contain DNA, RNA or microRNA (miRNA), which can induce endothelial regeneration by promoting proliferation of mature ECs (Deregibus et al., 2007).

Jansen et al. (2013) showed a protective effect of EMPs in murine models. It was demonstrated that injected EMPs accelerate endothelial repair after electric endothelial denudation of carotid artery.

5. Nutraceutical effects in vascular ageing

Several studies have already demonstrated that both in animal models and in humans, dietary intervention can positively modulate ageing process, affecting parameters related to inflammageing (Santoro et al., 2014). Also EPCs seem to be influenced by diet, although, it is not clearly explored.

The CVDs are mostly attributable to lifestyle. In Western world, it is often sedentary, including a diet rich in saturated fat and sugars, with an altered omega-3 or omega-6 fatty acids ratio, fruits, vegetables and fibers (Vasto et al., 2014). On the contrary, a reduction in the onset of CVDs is observed in Southern Europe. This event could be attributable to health-promoting dietary patterns, such as MD, rich in nutraceuticals (Estruch et al., 2013).

These molecules, mainly, have antioxidant and anti-inflammatory properties that confer to MD anti-ageing features (Vasto et al., 2014; Visioli and Galli, 2001). Resveratrol has anti-inflammatory effects; oleic acid, lycopene, quercetin, anthocyanins and catechins, all powerful antioxidants, are able to reduce the oxidation of low density lipoprotein (LDL) or, more generally, to have cardioprotective effects (Estruch et al., 2013) (see Table 1).

The main and common food in Mediterranean basin is the extra virgin olive oil (EVOO), subject matter of many researches thanks to its strong nutraceutical properties (Aiello et al., 2015; Virruso et al., 2014).

A large intervention trial showed that a diet rich in EVOO or in nuts could reduce the risk of CVDs by 30%, compared to the low fat counterparts (Perez-Martinez et al., 2007). This means that a diet rich in unsatured fat is healthier than a low fat one.

A systematic review showed that the adherence to MD is associated with positive effects in endothelial function as well as in inflammation (West, 2001). The explanation is a decrease of oxidative stress due to the antioxidant foods, mainly EVOO (Visioli and Galli, 2001).

Moreover, our pilot study on table green olives, conducted on healthy volunteers, demonstrated a decrease of oxidative stress and an anti-inflammatory effect, highlighted by a reduction of malondialdehyde and IL-6 levels, respectively (Accardi et al., in press).

Data show that EVOO decreases the pro-inflammatory environment, induced by oxidized LDL in ECs. With its high content in monounsaturated fatty acids (MUFA) and polyphenols, EVOO might exert beneficial effects in the development and progression of age-related diseases. In fact, its non-saponifiable portion (1–2%) contains about 230 bioactive components, such as carotenoids, mainly lycopene, sterols; and the phenolic compounds, such as oleoeuropein, oleocanthal, hydroxytyrosol and tyrosol with antioxidant and anti-inflammatory properties (Aiello et al., 2015; Virruso et al., 2014). Both *in vitro* and *in vivo* studies demonstrated the endothelium-protective properties of these molecules (Frankel, 2011; Martín et al., 2010; Moreno-Luna et al., 2012; Schwingshackl et al., 2015; Virruso et al., 2014). Different trials showed that polyphenol intake has been associated with low mortality rates caused by coronary heart disease (Hertog et al., 1995; Horn et al., 2014). These compounds are able to bind LDL, increasing resistance to oxidation and acting as radical scavenger (Gimeno et al., 2007). Moreover, it was seen that the consumption of polyphenol-rich olive oil can decrease blood pressure (BP) and improve endothelial function in young women with high-normal BP (Moreno-Luna et al., 2012). The C Reactive Protein and IL-6 levels, as well as the ones of flow-mediated dilatation and E-Selectin that characterize endothelial function are positively modulated after dietary interventions with EVOO (Schwingshackl and Hoffmann, 2014).

About its anti-inflammatory properties, hydroxytyrosol and oleocanthal have ibuprofen-like activity, inhibiting the cyclooxygenases 1 and 2, responsible for prostaglandin production. Moreover, polyphenols inhibit NF-κB pathway that leads to the expression

Table 1
Nutraceuticals and their functions. This table shows the main nutraceuticals present in Mediterranean foods. The principal sources are red fruits and vegetables, as well as olives and extra virgin olive oil.

Molecule	Origin	Function	Family	Ref.
Resveratrol	Grape skin, blackberry, blueberry, nut, dark chocolate	Cardiovascular protection; antioxidant by activation of NRF-2; anti-inflammatory; increase eNO production; reduction of oxLDL and systolic and diastolic BP	Polyphenol	Ndiaye et al. (2003); Ungvari et al. (2010)
Lycopene	Tomato, red carrot, strawberry, cherry, asparagus, olive, watermelon	Anti-inflammatory in atherosclerosis process; antioxidant; BP reduction; reduction of oxLDL	Carotenoid	Burton-Freeman et al. (2012); Paran et al. (2009)
Oleic acid	Olive, nut, green olive	Reduction of oxLDL	Lipid	Schwingshackl and Hoffmann, (2014)
Quercetin	Apple, red onion, caper	Antioxidant	Flavonoid	Horn et al. (2014); Malavolta et al. (2015)
Catechin	Green tea, cocoa	Antioxidant	Flavonoid	Camargo et al. (2012); Hertog et al. (1995); Horn et al. (2014)
Anthocyanin	Red-purple fruit and vegetables	Antioxidant; cardioprotective; neuroprotective; photoprotective	Flavonoid	Horn et al. (2014); Malavolta et al. (2015)
Oleuropein	Olive, EVOO	Radical scavenger, blocking the LDL oxidation	Polyphenol	Gimeno et al. (2007)
Hydrossityrosol	Olive, EVOO	Inhibition of COX-1 and COX-2	Polyphenol	Martín et al. (2010)
Oleocanthal	Olive, EVOO	Inhibition of COX-1 and COX-2	Polyphenol	Virruso et al. (2014)

of pro-inflammatory genes (Beauchamp et al., 2005; Romier et al., 2008; Zhang et al., 2009).

Another potential health-promoting component of EVOO is oleic acid. It is the main MUFA of EVOO and is claimed to increase the resistance of LDL to oxidation. It has also been suggested that MUFA can decrease all-cause (11%) and cardiovascular mortality (12%), cardiovascular events (9%), as well as stroke (17%) (Schwingshackl et al., 2015).

Moreover, oleic acid suppresses cytotoxic function of natural killer cells with a consequent anti-inflammatory effect (Yaqoob et al., 1994). In general, unsaturated fatty acids improve endothelial function decreasing intercellular adhesion molecule (ICAM)-1 production by ECs and reducing leukocyte adhesion (Bemelmans et al., 2002).

But, not only EVOO has to take into account as healthy food. In fact, many antioxidants, not necessary belonging to MD, have been studied, showing cardioprotective properties.

New interesting effects were proposed about quercetin, a flavonoid that is ubiquitously present in MD, in a variety of fruits and vegetables. It has been traditionally viewed as a potent antioxidant and anti-inflammatory molecule. In addition, the hypothesis is that in pre-senescent and senescent human cells, this bioactive molecule has senolytic effects. Its action provides a sort of rejuvenation of cells because promotes the selective killing of senescent cells, while permits the expansion of younger cells, lacking senescent markers (Malavolta et al., 2015). *In vivo* studies on mice demonstrated a possible effect of the combination of quercetin with the anti-tumor drug desatinib in the improvement of vascular endothelium damage, representing a possible therapeutic approach to ameliorate cardiovascular function (Zhu et al., 2015).

Further, flavonoids, such as anthoxanthins, especially flavones and flavonols, present in red wine, vegetables and fruit, as well as in tea, soy and cocoa, have antioxidant properties. They exert the cardioprotective effect, mediating vascular relaxation (Kyaw et al., 2004).

Flavonols present in cocoa can improve endothelial function, as well as can lower the level of markers of endothelial integrity, vascular cell adhesion molecule (VCAM)-1 and flow mediated dilatation, in healthy subjects and patients with coronary artery disease (Hertog et al., 1995; Horn et al., 2014). The flavonoids act on AMP-activated protein kinase (AMPK) pathway, which is activated when intracellular ATP levels decrease. This signaling is phylogenetically conserved and is the guardian of systemic and cellular energy status. Vascular AMPK seems to be involved in the regulation of vascular tone. In case of hypoxia, it can induce relaxation of big vessels. In fact, its activation phosphorilates eNOS, acting as regulator of blood stream (Davis et al., 2006).

The resveratrol, polyphenol found in grape skins so in red wine, other grape skin derived products, lycopene and carotenoids in general, can alleviate the inflammation in cardiovascular degenerative processes, such as atherosclerosis. They and cocoa also have antioxidant effects, increasing the NO endothelial release by the activation of NOS, including the production of smooth muscle relaxation through GMP-mediated pathway with a reduction of systolic and diastolic BP. In addition, *in vitro* and *in vivo* studies have suggested that resveratrol has effects on the modulation of the EPC levels. This molecule seems to modulate the nuclear factor-

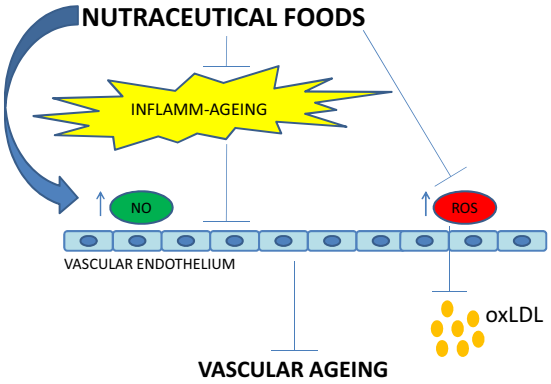

Fig. 1. Effect of nutraceutical foods on vascular endothelium. Nutraceutical foods may reduce inflammageing because their high content in anti-inflammatory and anti-oxidant molecules. Consequently, inflammageing lowers its effect on endothelium, delaying the vessels' ageing process by increasing nitric oxide bioavailability with a positive effect of vasorelaxation on the wall and diminishing ROS hyperproduction so reducing LDL oxidation. ROS: reactive oxigen species; NO: nitric oxide; oxLDL: oxidized low density lipoprotein.

E_2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway In the cellular nucleus, it binds the ARE sequence, permitting the expression of antioxidant enzymes, such as NQO1-NADPH quinine oxidoreductase 1 and hemeoxigenase 1. Moreover, it controls the expression of gamma glutamyl cysteine synthetase, enzyme involved in the synthesis of glutathione. The consequence is an endothelial protective effect, dependent on a dose presumably achievable in human by resveratrol supplementation (Ungvari et al., 2010, 2008).

Furthermore, the consumption of tomato extract, containing lycopene and beta-carotene, results in a decrease of LDL oxidation, induced by high-fat meal and the one of IL-6 (Burton-Freeman et al., 2012; Camargo et al., 2012; Ndiaye et al., 2003; Paran et al., 2009). The reduced oxidation of LDL also is an effect of flavonoids. They determine an inhibition of lectin-like oxidized receptor-1 (LOX-1) that binds oxidized LDL. LOX-1 is expressed in pro-atherogenic settings, such as hypertension or hyperlipidemia, and is accumulated in the atherosclerotic lesions. It reduces the release of NO and increases the expression of adhesion molecules and inflammatory cytokines. So, its inhibition results in vasorelaxation and in a possible prevention or treatment of CVDs (Nishizuka et al., 2011).

See Fig. 1 for an overview.

6. New insights in vascular ageing

The effect of MD on vascular ageing is not only due to bioactive compounds, since the low animal proteins intake of the diet could contribute to the protection of endothelium (Vasto et al., 2014).

In general, meat is a source, and sometimes, the main source of proteins in different diets worldwide. Nevertheless, many studies demonstrated that its great consumption is correlated with CVDs, type 2 diabetes and cancer, in particular the colorectal one (Rohrmann et al., 2013).

Protein restriction (5% to 15% intake) increases life span in rodents up to one third compared to a high-protein diet (50% intake) (Solon-Biet et al., 2014). The molecular reason was explained, taking into account their action on the mechanistic target of rapamycin (mTOR) pathway downregulation. mTOR is a serine-threonine protein kinase, belonging to the phosphatidylinositol-3OH kinase related family. This kinase is a nutrient/hormone-sensing molecule that regulates cellular growth and metabolism, with mitogenic effects due to the promotion of protein synthesis and mRNA translation. Its down-regulation, possibly related to dietary restriction, in terms of calories and animal proteins intake, leads to mTOR complex 1 inhibition, favoring longevity. Thus, high proteins intake is a possible responsible of accelerated ageing in model organisms. However, recent studies demonstrated that an aminoacids restriction is powerful in life-span extension, more than a total protein restriction. In fact, Mirisola et al. (2014) demonstrated that in yeast, the threonine and the valine, in particular, promote ageing *via* Tor-1 activation, the orthologue of mTOR in yeast. The main responsible aminoacid involved in ageing damages seems to be the methionine. Rats fed with a high methionine diet (2%) compared with ones fed with high-protein diet (50%) showed accelerated vascular ageing. They had decreased aortic wall elasticity so they were more prone to hypertensive consequences. Their aorta smooth muscle cells were degenerate and with necrosis and they were replaced by chondroid cells and foci of fibrosis. Methionine, in particular, could be the explanation for the damages induced by red meats. In fact, vegetable proteins are less rich of methionine and sulfur aminoacids, in general, than the animal ones (Fau et al., 1988; Longo et al., 2015).

Comparing the consumption of red meat with one of poultry and dairy products, as well as nuts, it was demonstrated a higher rate of stroke in the first case. The same was observed for coronary heart disease (Bernstein et al., 2012; Bernstein et al., 2010).

Moreover, many studies provide evidences about the effects of miRNAs, likely able to regulate endothelial function and inflammation. miRNAs are small endogenous non-coding genes present in animals and plants. They modulate mRNA gene expression in a post-transcriptional manner. miR-146a plays a role in inflammation, cell senescence and ageing. It seems to block Toll-like receptor 4 and cytokines production and could represent a possible marker of senescence-associated pro-inflammatory status in cell involved in vascular remodeling (Brudecki et al., 2013; Olivieri et al., 2013; Vasa-Nicotera et al., 2011).

In addition, a number of studies investigated whether miRNAs can be modulated by food.

In a PREDIMED trial, it was shown a novel association between miRNA and stroke incidence by the modulation of triglyceride levels (Corella and Ordovás, 2014).

The anti-inflammatory properties of some polyphenolic compounds have shown effects on them *in vitro*. miRNA 126, selectively expressed in ECs, decreases the mRNA expression of VCAM-1 (Harris et al., 2008). This effect was confirmed in a study on Human Umbilical Vein Endothelial Cells (HUVEC), stimulated with lipopolysaccharide and treated with a polyphenol extracted from grape (Noratto et al., 2011). Conversely, another study in HUVEC, treated with curcuminoids, showed that their anti-inflammatory effects modulate VCAM-1 expression, but it did not involve miRNA 126 (Angel-Morales et al., 2012).

Moreover, the expression of miR-221 and 181 seems to be modulated by flavanol-metabolites in ECs (Milenkovic, 2014).

So, although it is not completely clear the exact role of miRNAs in vascular ageing, they represent a new interesting insight in vascular ageing process. They represent new field of research with interesting implications in human development and diseases.

Some evidences demonstrated the interrelationship between miRNA expression, DNA methylation, and histone modifications, so molecular biology techniques, such as over-expression or selective knockout of some miRNAs could represent a therapy in the treatment of CDVs (Corella and Ordovás, 2014).

7. Conclusions

During life, different stimuli, such as drugs, oxidative stress and a general functional decline of physiological processes, can reduce the regenerative capacities of EPCs and can induce an impairment of endothelial function, creating a negative feedback loop

for ageing. Since these progenitors preserve vascular endothelium homeostasis and participate to re-endothelialization and neo-vascularization, they can represent a possible target in the treatment of vascular ageing.

The possibility to modulate the number and the functional activity of EPCs by nutritional therapy, could be useful in the treatment and in the prevention of age-related diseases, such as CVDs. Nowadays, several studies focused on beneficial effects of nutraceuticals. The presence of these bioactive molecules in foods typical of MD, confers healthy properties to this nutritional pattern. MD fruits and vegetables, increasing antioxidant state, improve endothelial function and modulate inflammation. Thus, they may enhance the regenerative capacity of the endothelium. In fact, this diet decreases ROS production and, so, reduces endothelial damage, determining lower apoptosis rate in ECs and protecting them from senescence. Furthermore, MD induces NO release, resulting in a vasorelaxation, and ameliorate endothelial function. This seems to be associated with a greater regeneration capacity and with an increase of circulating EPCs. Moreover, *in vitro* studies demonstrated that antioxidant bioactive molecules from olive oil may decrease oxidative stress by the modulation of Nrf2-heme oxygenase-1 pathway that restore the EPCs function and reduce the number of senescent cells.

Thus, nutraceuticals act not only direct by their antioxidant or anti-inflammatory properties but also indirectly, *via* the modulation on molecular pathways that improve the expression of genes involved in anti-ageing mechanisms.

In conclusion, it is possible to speculate that the key of the healthy effect of MD on EPCs is not narrow in a single component or in a single food but in a synergic or additive effect of the overall dietary pattern. Therefore, further studies need to be carried out to fully understand the specific role of Mediterranean nutraceutical foods played on vascular ageing.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' contribution

Dr. Giulia Accardi, Dr. Anna Aiello and Dr. Caterina Maria Gambino were involved in drafting the paper. Prof. Calogero Caruso and Prof. Giuseppina Candore gave the final approval of the version to be published. All authors read and approved the final paper.

Giulia Accardi is a Post Doc in Pathobiology. Anna Aiello, Caterina Maria Gambino and Claudia Virruso are students of the PhD course directed by Calogero Caruso.

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V. Nutrient sensing pathways as therapeutic targets for healthy ageing (*in revision*)

Nutrient sensing pathways as therapeutic targets for healthy ageing

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Nutrient sensing pathways as therapeutic targets for healthy ageing

Abstract

Introduction: In the present paper, the authors have discussed anti-ageing strategies which aim to slow the ageing process and to delay the onset of age-related diseases, focusing on nutrient sensing pathways (NSPs) as therapeutic targets. Indeed, several studies have already demonstrated that both in animal models and humans, dietary interventions might have a positive impact on the ageing process through the modulation of these pathways.

Areas Covered: Achieving healthy ageing is the main challenge of the 21th century because — lifespan is increasing, but not in tandem with good health. The authors have illustrated different approaches that can act on NSPs, modulating the rate of the ageing process.

Expert Opinion: Humanity's lasting dream is to reverse or, at least, postpone ageing. In recent years, increasing attention has been devoted to anti-ageing therapies. The subject is very popular among the general public, whose imagination runs wild with all the possible tools to delay ageing and to gain immortality. Some approaches discussed in the present review should be able to substantially slow down the ageing process, extending our productive, youthful lives, without frailty.

Keywords: Ageing; anti-ageing approaches; dietary patterns; nutraceuticals; nutrient sensing pathways

1. Introduction: overview of the ageing process

Healthy ageing is the main challenge of the 21th century both in Western and developing countries.[1] To develop preventive and therapeutic measures that can support healthy lifespan, an integrative approach is needed.[2] To do it, it is important to change daily habits, and diet is, probably, the most important one.[3,4]

Ageing is a complex process which decreases the ability to adapt to stress, causing a decline in functional capacity. This event, characterized by a progressive loss of the physiological integrity of many interrelated systems, leading to impaired function and increased vulnerability to disease and likelihood of death. In May 2012, a group of scientists and clinicians developed a consensus statement to highlight the importance of a common view on ageing and healthy lifespan. As reported in the panel: "Ageing processes are defined as those that amplify the vulnerability of subjects, as they became older, to the factors that finally lead to death. Individuals with the same chronological age may have different rate of ageing and different biological age".[5]

Ageing is a physiological and ineluctable process, characterized by many processes and molecular dysfunctions, whose burden falls into two different phenotypes: successful and unsuccessful. The latter is manifested by those that develop one or more age-related diseases after reaching 65 years of age. Conversely, successful ageing involves avoidance or delayed onset of pathologies, including cardiovascular diseases, which are the main cause of death, and other organ specific diseases, in addition to disability, preservation of desirable cognitive, physical function and social activities throughout the lifespan.[5,6]

Ageing is accompanied by a chronic low grade inflammatory state, known as inflammageing, a term derived from a combination of inflammation and ageing.[7,8] In general, inflammation is not a negative response because it serves to protect the organism from harmful conditions and contributes to healing process. On the contrary, at a later stage of life, the chronic inflammatory stimuli can lead to detrimental phenomena that increase the risk of diseases and death. According to the theory of antagonistic pleiotropy, in early life, when natural selection is

strong, the inflammation should be beneficial but becomes dangerous during ageing when selection is weak.[9]

A wide range of different stimuli contribute to develop inflammageing, however some factors can impact inflammageing, counteracting it, such as an anti-inflammatory diet and physical activity (see Figure 1).[3,10,11]

Increased inflammation is mainly attributed to a chronic lifelong antigenic stimulation, unforeseen by evolution. So, inflammageing may derive from age-related changes to the immune system, so called immunosenescence. In line with the remodelling theory of ageing [12], in immunosenescence, innate immunity is largely conserved or even up-regulated, while clonotypic immunity deteriorates with age. This causes reduced ability to clear novel pathogens and T-cell populations, responsible for the production of pro-inflammatory cytokines, are amplified.[13,14] Paradoxically, centenarians show high levels of pro-inflammatory markers, counterbalanced however by anti-inflammatory ones.[15]

Other causes of inflammageing can be represented by damaged macromolecules and endogenous host-derived cell debris that, accumulating with age, as a consequence of their increased production and/or inadequate elimination, are sources of chronic damage.[7] The amount of senescent cells increases with age and can have deleterious effects on tissues, altering the function of nearby cells. This is due to the acquisition of the senescence-associated secretory phenotype (SASP) that induces senescent cells to produce pro-inflammatory mediators.[16] Furthermore, visceral obesity is known to be associated with a pro-inflammatory status.[17]

Moreover, harmful metabolites, produced by the microbiota, likely participate in the ageing process. Although the intestinal microbiota is relatively stable throughout adulthood, age-related changes in the gastro-intestinal tract, as well as changes in diet and host immunity reactivity, inevitably affect the composition of bacterial populations. In the gut, prolonged retention time leads to an increase in bacterial protein fermentation and consequently putrefactive processes with a greater susceptibility to inflammatory diseases.[18].

Changes with age in specific bacterial genera and species have been identified, with a considerable inter-individual variation that continues into old age. Many studies have demonstrated a decline, with age, in both the total number and in the species diversity of *Bacteroides* and *Bifidobacteria.* High fat consumption produces a decrease in the number of *Bifidobacteria*. This group of bacteria has been shown to reduce lipopolysaccharide (LPS) levels in mice and to improve the mucosal barrier function. Interestingly, *Bifidobacteria* do not degrade intestinal mucous glycoproteins like other pathogenic bacteria do. This effect promotes a healthier microvillus environment by preventing permeability and bacterial translocation. Some studies provide evidence that the processes involved in the development of endotoxemia that causes low-grade inflammation and the corresponding metabolic disorders in response to high fat consumption are associated with an increased intestinal permeability, reducing the expression of genes coding for tight junction proteins (see Figure 1).[19-22]

In addition, age-related changes in microbial populations would lead to a decline in the production of short chain fatty acids (SCFAs). In non-aged people, fibres present in vegetables, fruits, whole cereals, and other fermentable foods are converted ,in the colon, into SCFAs by microbiota, with the production of acetate, propionate and butyrate, that have positive effects on health.[23] It was speculated that this reduction of SCFAs in elderly faeces, likely related to the reduction of *Bacteroides* and *Bifidobacteria*, might contribute to an adverse effect on immune function.[18] In fact, the decrease of *Bifidobacteria* observed in elderly was associated with a decreased inhibition of the growth of some pathogens and, potentially, with an increase in susceptibility to infection.[24] However, it is noteworthy that, on the basis of studies in both animals and humans, dietary intake appears to be a major short and long term regulator of the structure and function of gut microbiota.[25]

Inflammageing, with its close links to the oxidative stress that increases the release of inflammatory mediators, represents one of the most important triggers of age-related diseases, like

Alzheimers, cardiovascular diseases, type 2 diabetes mellitus (T2DM), sarcopenia, cancer, metabolic syndrome, and frailty.[10]

Moreover, currently available evidence suggests that anabolic signalling accelerates ageing and age-related diseases, whereas decreased nutrient signalling extends survival and longevity. The process is, at least in part, linked to the modulation of downstream nutrient sensing pathways (NSPs), such as the activation of forkhead box O (FOXO) 3A or the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The activation of the former counteracts the oxidative stress through the transcriptional activation of homeostatic genes. The inhibition of the latter one switches off the transcription of inflammatory genes, thus slowing down the inflammaging process.[2,26-29]

2. NSPs: key role in healthy ageing

For many years, biogerontologists have investigated possible interventions to slow the rate of ageing and increase lifespan. Nowadays, the findings that diet can contribute to successful ageing has created new and interesting possibilities for anti-ageing medicine.[4,11,30]

In the present paper, we do not need to distinguish between ageing and age-related diseases because, as discussed by Blagosklonny, in protected environments, humans and animals die from age-related diseases, which are a manifestation of ageing. [31,32] Since human longevity is limited by death from age-related diseases, a true anti-ageing drug must delay the onset of age-related diseases. In other words, unless a drug delays age-related diseases, it will not extend lifespan. And vice versa, if a drug prevents age-related diseases, it must extend lifespan. See, however, Blumenthal for a comprehensive critical review of the relationship between ageing and disease.[33]

Several studies have suggested that both in animal models and humans, dietary intervention can prevent or decrease various age-related diseases, by positively regulating ageing process through the modulation of NSPs such as the insulin/insulin-like growth factor (IGF)-1, the mechanistic (previously referred to as mammalian) target of rapamycin (mTOR) and the sirtuin

pathways.[34-37] These are activated by nutrients, such as carbohydrates or proteins that trigger signals which result in a downstream activation of genes involved in ageing process. An excessive intake of nutrients however can accelerate these events and increase the risk of unsuccessful ageing. In fact, the activation of these metabolic pathways is characterized by inflammation and mitochondrial dysfunction, with an increase of oxidative stress and a reduction of autophagy. This is a self-degradative process, important for the balance of sources of energy in development and in the response to nutrient stress [26] that, in case of reduction, promote inflammageing and unsuccessful ageing.

Through the downregulation of IGF-1 and mTOR cascade or the up-regulation of sirtuins one can extend lifespan in various model organisms, including mammals.[26] These effects are also obtained by the presence of specific single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in NSPs, such as IGF-1 receptor (IGF-1R) and FOXO3A.[38]

Thus, dietary and pharmacological interventions relating to these pathways might delay or prevent age-related pathologies and improve the quality of life during ageing.

2.1 Insulin/IGF-1 pathway

The Insulin/IGF-1 pathway is evolutionarily conserved (although with an obvious increase in its complexity from yeast to human) and multiple genetic manipulations, that attenuate signalling intensity at different levels of the pathway, seem to extend the lifespan of model animals.[26]

The insulin/IGF-1 signalling cascade starts from the binding of insulin or IGF-1 to the insulin or IGF-1 receptors. Consequently, the intracellular substrate proteins, known as insulin responsive substrates (IRSs), act as mediators, binding specific Src-homology-2 domain proteins. These include enzymes, such as phosphatidylinositol 3-kinase (PI3K) and other intracellular signalling molecules, such as the adaptor protein growth factor receptor-bound protein 2, which is connected with the Rat sarcoma protein pathway, a mitosis stimulating proto-oncogene. PI3K activates via the second messenger phosphatidyl inositol 3-phosphate, leading to the activation of

protein kinase B (PKB, also known as AKT), that acts on glycogen synthase kinase 3 involved in cellular glucose uptake. Moreover, it stimulates the NF-κB pathway, involved in immune-inflammatory processes. In addition, this pathway, through the kinases PI3K and AKT, activates mTOR complex.[39,40]

Downstream, the insulin/IGF-1 pathway modulates the expression of transcription factors, like FOXO3A, extensively studied for its role in ageing and longevity. It is one of the orthologue of daf-16 in *Caenorhabditis* (*C.*) *elegans* and is involved in stress resistance and survival, through its action on homeostatic genes.[26] Some FOXO3A SNPs were associated with longevity in different populations. This highlights the role of FOXO3A in successful ageing, probably due to an increased expression of FOXO3A.[38,41] In humans, ageing is associated with lower levels of circulating IGF-1, and in long-lived people IGF-1R was correlated with modulation of lifespan through the attenuation of its signalling.[38,41] However, growth hormone (GH) and IGF-1 levels decline both in successful and unsuccessful ageing, whereas in models such as nematodes and mice, a constitutively decreased signalling pathway extends longevity. It has been suggested that a constitutively reduced signalling implies a lower rate of cell growth and metabolism, hence a lower rates of cellular damage. On the other hand, extremely low levels of signalling are incompatible with life, as shown by embryonic death of mice with null mutation in genes downstream in the pathway.[42]

GH is secreted from the anterior pituitary gland in response to stimulation by GH releasing hormone, ghrelin, and dietary components such as proteins, whereas its secretion is inhibited by IGF-1, somatostatin and insulin. GH stimulates the hepatic production of IGF-1, and acts on insulin/IGF-1 pathway, modulating insulin sensitivity.[27] Moreover, it does not directly interact with the insulin receptor but, in experimental models, it was seen that an excess of GH might be associated with hyperinsulinemia. On the contrary, the GH deficiency is associated with increased insulin sensitivity and decreased insulin secretion and fasting blood glucose concentration. GH and IGF-1 (i.e., the somatotropic axis), synergistically, act on carbohydrate metabolism and their

signals, although the sequence of molecular events has not clarified, converge with that of insulin. In particular, GH stimulates lipolysis and inhibits insulin-induced suppression of hepatic gluconeogenesis. These counteract insulin action and reduce the need for a dietary source of carbohydrates. IGF-I plays an important role in carbohydrate metabolism, maintaining a balance between the actions of insulin and GH. IGF-I also participates in the control of insulin sensitivity and action and plays an important role in the hormonal balance between GH and insulin in addition to its effect on reducing circulating GH levels.[43]

People with impaired GH receptor (Laron Syndrome) have very low incidence of age-related diseases. It was shown that humans with GH receptor deficiency are characterized by a reduction of IGF-1 and insulin levels in serum, displaying no diabetes mortality.[44,45]

So, continuous activation of these anabolic pathways by nutrients, i.e., proteins (GH and IGF-1) and sugars (insulin) with high glycaemic index (GI), is responsible for accelerating ageing and age-related diseases, such as cancer, stimulating inflammation and cell proliferation, and decreasing homeostatic responses (see Figure 1).

However, the criticism of a recent review, according to which, available data do not support the hypothesis of the role of somatotropic axis in modulating ageing and longevity in humans, should be taken into account. The article argues that, because the life-history strategies of humans are very different from those of models, this hypothesis is unwarranted.[46]

2.2 mTOR pathway

TOR is a serine/threonine kinase belonging to the phosphatidylinositol kinase-related family and it is structurally as well as functionally highly conserved throughout eukaryotes.

Yeast, in contrast to higher eukaryotes, which have a single TOR gene (mTOR), has two TOR genes: TOR1 and TOR2.[47,48] Two different TOR containing complexes are present in yeast cells: the rapamycin-sensitive (for the pharmacological use of rapamycin see paragraph 3.3) TOR complex 1 (TORC1) and the rapamycin-insensitive TOR complex 2 (TORC2).[49] These

complexes respond to nutrients, especially some amino acids, such as methionine, glutamine, threonine and valine as crucial activators of TOR signalling. Mutations in genes relevant to glutamine metabolism alter TOR activity and valine/threonine restriction impairs phosphorylation of the major TOR target, Sch9 kinase, increasing lifespan.[50,51]

However, TORC1 and TORC2 have non-overlapping cellular functions. TORC1 mainly regulates growth, metabolism, and stress response through regulation of transcription, translation and autophagy. TORC2 mainly regulates actin cytoskeletal remodelling.[52] Decreased activity of the TORC1 signalling pathway due to mutations, drugs or amino acids depletion, increases chronological lifespan in yeast and improves resistance to stresses through relocalization of transcription factors. Genetic down-regulation of TORC1 activity in yeast, as well as in worms and flies, extends longevity.[50,51]

In mammals, mTOR participates to the formation of mTORC1 and mTORC2. mTORC1 promotes anabolic processes and blocks catabolism and autophagy, and its best known target is 70-kDa ribosomal protein S6 kinase 1 (p70S6K). Reduced growth factor signalling suppresses mTORC1 activity, leading to down-regulation of metabolism and promotion of survival, during starvation or intermittent fasting.[53] mTORC2 phosphorylates and activates PKB, suggesting a regulation on mTORC1.[54]

Stress conditions such as hypoxia and osmotic stress can also inhibit mTOR activity.[55,56] Instead, nutrients, especially amino acids and growth factors, such as IGF-1, activate mTOR pathway.[57]

Low cellular ATP levels or a high AMP/ATP ratio activates the cellular energy sensor 5' adenosine monophosphate-activated protein kinase (AMPK), also activated by caloric restriction (CR, see paragraph 3.1). AMPK, in turn, inhibits mTORC1, as well as creating genotoxic stress via p53, oxidizing agents, cigarette smoke and glucocorticoids that act via the induction of the hypoxia-inducible factor (HIF) DNA damage response 1.[57,58] Moreover, mTORC1 inhibits autophagy in

response to nutrients and growth factors, while upregulation of autophagy is observed during starvation or in response to oxidative stress.[59]

In adipose tissue, the activation of mTORC1 increases the number and size of adipocytes, while fasting, as well as rapamycin, leading to fat mass reduction. In addition, mTORC1 facilitates the accumulation of triglycerides, promoting adipogenesis as well as lipogenesis, and preventing lipolysis and β-oxidation.[60,61]

mTORC1 also seems to be an important regulator of mitochondria function. Inhibition of mTORC1 modifies mitochondrial phosphoproteome, reduces mitochondrial membrane potential, oxygen consumption and therefore cellular ATP levels and reactive oxygen species (ROS) production rate, which contribute to oxidative stress.[62]

So, the mTOR kinase, which is part of two complexes, mTORC1 and mTORC2, has a central role in the regulation of metabolism, integrating numerous environmental signals and indicating whether conditions are favourable for anabolic processes. In fact, this pathway responds to stimuli, including insulin and IGF-1, amino acids and glucose levels, cellular energy status and oxygen levels. These data again support the idea that intense anabolic activity is responsible for accelerating ageing and age-related diseases (see Figure 1).[28,42]

2.3 Sirtuins pathway

Sirtuins, orthologues of Sir2 yeast protein (where Sir stands for silent information regulator since it silences certain genes), are a family of histone deacetylase enzymes, involved in the regulation of cellular mechanisms, identified as anti-ageing molecules in model organisms. Their action was, in fact, linked to some cellular processes, such as cellular metabolism, apoptosis, DNA repair, development, inflammatory response and neuroprotection.[63]

The sirtuin role was first investigated in yeast, in which the overexpression of the SIR2 gene leads to an increased lifespan. In *Saccharomyces* (*S.*) *cerevisiae*, Sir2 is involved in changes of

cellular metabolic pattern, in the improvement of the genomic stability and in the extension of the lifespan.[29,42,64]

Sirtuin activation seems to contribute to CR-induced longevity in model organisms, including, *S. cerevisiae*, *C. elegans* and *Drosophila* (*D.*) *melanogaster*. In fact, some studies showed that the induction of sirtuin activity could mimic the beneficial effects of CR, reducing insulin/IGF-1 downstream signalling and decreasing proteins ubiquitination. Moreover, CR, in itself, up regulates Sir2.[65-67]

In mammals, there are seven yeast homolog sirtuins (SIRT1-SIRT7). SIRT1, 6, and 7 are localized in the nucleus; SIRT3, 4 and 5 in the mitochondria and SIRT2 in the cytosol. No clear data exist about SIRT5 and 7, activated by p53. All sirtuins contain 275-aminoacids catalytic subunits and use NAD^+ as a co-substrate. It is converted to nicotinamide, whose concentration is determined by the nutritional state of the cell.[42]

In particular, in humans, SIRT1, 2, 3, and 6 are activated by CR, while it inhibits SIRT4. SIRT1 modulates responses to oxidative stress by FOXO3A deacetylation. In addition, it has a protective role in endothelial function, preventing cardiovascular diseases. Similarly, SIRT3 is implicated in metabolism and mitochondrial function, including ROS detoxification. Moreover, a study on the Italian population revealed a correlation between a high rate of its activity and longevity.[29,68] On the contrary, the overexpression of SIRT7 is observed mainly in cancer cells, while its depletion contributes to prevention of ageing. Whereas SIRT2, especially active in the cytoplasm, has anti-inflammatory effects, in activating NF-κB.[29,69] Recently, SIRT6 has been identified as a critical regulator of transcription, genome stability, telomere integrity, DNA repair, and metabolic homeostasis, with an important effect on the ageing process.[70]

So, sirtuins, which sense low energy states by detecting high NAD^+ levels, acts as transcriptional effectors by controlling the acetylation state of histones, signalling nutrient scarcity and catabolism.[71] Thus, their action is in opposition to the two previous NSPs, hence favouring healthy ageing and longevity (see Figure 1).[42]

3. Therapeutic interventions to modulate NSPs

To delay ageing and to achieve successful ageing it is necessary to identify new therapeutic targets. As we illustrated, NSPs are one of these and different approaches are possible to modulate them (see Figure 1).

3.1 Dietary intervention approaches

In model organisms and humans, the possibility to modulate ageing, with a non-invasive method, has been identified in the dietary restriction (DR), defined as "Dietary regimen in which specific food groups or micronutrients are reduced or removed from the diet".[72] Accordingly, a clinical trial demonstrated that an increased consumption of low GI fruits improves glycaemic control among people with diabetes and low GI foods are related to a reduction in the risk of coronary heart diseases and T2DM. Moreover, foods rich in soluble fibres slow glucose absorption and, consequently, the subsequent insulin response.[73-75]

The most well defined dietary intervention to delay ageing is CR. It is a process involved in lifespan extension, acknowledged for almost 80 years, consisting in the reduction of total calorie intake by 20-40% without malnutrition.[26,76] In models, CR leads to lifespan extension and involves the insulin/IGF-1 and mTOR signalling reduction, the modulation of glycaemia and insulinemia, as well as the increase of insulin sensitivity, and the activation of sirtuins pathways.[26,77,78] In humans, CR, combined with a low GI diet, has been shown to improve cardiovascular parameters and weight loss.[79]

On the other hand, on the basis of theoretical models, it has been predicted that the large increases in mean life span and maximum life span potential observed in laboratory rodents subject to caloric restriction will not been obtained in human beings.[80,81] However, recently, data accumulated from observational and randomized clinical trials indicate that, in humans, CR results in some of the same metabolic and molecular adaptations that have been shown to improve health and slow the accumulation of molecular damage in models of animal longevity. In particular, moderate CR in humans improves multiple metabolic and hormonal factors that are implicated in the pathogenesis of T2DM, cardiovascular diseases, and cancer, that lead to morbidity, disability and mortality.[82]

Alternative approaches that mimic CR are being investigated, i.e. intermittent (IF) and prolonged fasting (PF). IF involves caloric intake on every other day for long period of time; in PF only water is consumed for two or more consecutive days. Both IF and PF have been shown to extend lifespan in animal models. In particular, in mice, IF seems to prevent or delay age-related diseases, and, in humans, preliminary clinical studies seem promising. Concerning the mechanisms, CR and fasting promote resistance to damage, activating FOXO (and other transcription factors), down regulating insulin/IGF-1 and mTOR pathways, and up regulating sirtuin pathways.[2,83]

Studying deeper the benefits attributed to CR in more depth, the scientists argued that protein restriction (PR) or the restriction of particular amino acids is sufficient to reduce IGF-1 concentrations and mTOR signalling, more than the simple CR.[36,72,77] In fact, rats fed by soy proteins have a higher lifespan than those fed by casein.[84] In addition, in *D. melanogaster*, the effect of CR is lost in presence of essential amino acids.[85] In particular, methionine and tryptophan, contained more in animal than plant proteins, seem to have more detrimental effects than other amino acids, and their reduction in the diet of various model organisms, extends their lifespan, though an as yet unclear molecular process.[2,77] Nevertheless, a few studies were performed on the potential benefits of PR on the human ageing process and one of the main limitations is the age stratification of analysed groups. In a case-control study, an association

between decreased protein consumption, reduction in IGF-1 levels and cancer in 50-65 years people but not in older population was demonstrated, suggesting that, in elderly, a higher protein intake, mostly from vegetables, should be recommend.[11,86]

However, although CR and fasting may be desirable on account of their health benefits, they are severe dietary regimens and not always applicable. An alternative approach may be a close adherence to the Mediterranean Diet (MedDiet), which also includes healthy lifestyle.

The MedDiet is one of the most studied dietary patterns. It is an alimentary regimen with low GI and low animal protein intake. It contains phytochemical compounds found in vegetables, fruits, red wine, olive oil or nuts with anti-inflammatory and anti-oxidant effects. Moreover, it mimics a sort of CR. In fact, studies on Sicilian centenarians demonstrated that their diet contains about 1200-1300 Kilocalories.[3,87] One explanation of its pro-longevity properties is the inhibition of mTOR, the downregulation of insulin/IGF-1 cascade, and the upregulation of sirtuins by the energy sensors AMPK and NAD^+, the low GI, the low animal protein intake and, consequently, the low consumption of methionine and tryptophan.[4]

A similar alimentary pattern may be represented by Asiatic diets, in particular by diet of Okinawa, an island with an high prevalence of centenarians. The Okinawan diet is characterized by relatively high consumption of unrefined, low GI sugars from vegetables, legumes and fruits, with moderate fish and marine food consumption.[88,89]

So, these diets represent an interesting starting point to delineate new or revised dietary patterns to reduce the activation of insulin/IGF-1 and mTOR pathways and, consequently, to extend healthy lifespan.

3.2 Nutraceutical approaches

Nutraceuticals are defined as “Naturally derived bioactive compounds found in foods, dietary supplements and herbal products, that have health promoting, disease preventing, or medicinal properties”.[90] These are constituents of different dietary patterns, such as the

Mediterranean and the Asiatic diets, and can modulate NSPs.[30] They explicate their action as hormetins, activating cellular stress response pathways, like the nuclear factor erythroid 2-related factor 2 (Nrf2), and leading to the transcription of antioxidant genes.[91]

In two clinical trials, it was seen that US men and women that consumed ~~a~~ large amount of fruit with purple skin, containing anthocyanidins (plant pigment belonged to flavonoids family), such as blueberries, grapes and strawberries, had minor risk of T2DM. In general, human intervention trials have shown that berries significantly improve insulin sensitivity, reduce fasting plasma glucose and the postprandial glucose response to a sucrose load. *In vitro*, the anthocyanidins interact with molecular targets and affect signalling pathways like NF-κB, reducing inflammageing. Moreover, in an animal model of T2DM, anthocyanins significantly decreased blood glucose concentrations and improved insulin sensitivity. In diabetic mice, bilberry extract reduces glucose production in the liver, acting on AMPK that increases the glucose uptake and its utilization in white adipose tissue and skeletal muscle.[92-94]

Resveratrol, an antioxidant polyphenol contained in grape skin, induces the down regulation of IGF-1 signalling in mice consuming a high-fat diet.[95] Interestingly, it induces apoptosis in human colonic cancer cells, even in presence of IGF-1, used in combination with other grape extracts.[96] In addition, it belongs to a group of substances that inhibit mTOR, as well as: ~~the~~ quercetin, a flavonoid found in fruits and vegetables, like apples and capers, the polyphenol epigallocatechin gallate, present in green tea, and the ethylxanthine caffeine, in coffee.[4] In particular, it was seen that quercetin, mainly present in tea, onions, red grapes, and apples, acts as anti-cancer agent by the inhibition of mTOR signalling, activating AMPK.[97]

Moreover, resveratrol is a part of a class of SIRT1-activating compounds (STACs), plant-derived metabolites such as flavones, stilbenes, chalcones and anthocyanidins that directly activate SIRT1 *in vitro* through an apparent allosteric mechanism. In mice, STACs activate SIRT1, binding it to a conserved N-terminal domain.[2,98] The discovery of natural STACs prompted the production of synthetic SIRT1 activators that are considerably more potent, soluble, and

bioavailable. In many *in vitro* and model organisms studies, resveratrol and synthetic STACs mimic the anti-ageing properties of CR, inducing gene expression changes and, *in vivo*, extending lifespan. [2,99] In addition, third generation STACs molecules have been discovered. Compared to resveratrol, these show more specificity and sensitivity and are much more potent. They include benzimidazoles, thiazolopyridines, and urea- ased scaffolds classes.[98]

Flavonols, contained particularly in cocoa products, indirectly act on insulin sensitivity with a particular effect on the vascular endothelium, promoting nitric oxide production, with anti-atherogenic and anti-thrombotic functions.[100,101] Moreover, isoflavones, another class of flavonol compounds, plentiful in soybeans, have inhibitory effect on mTOR pathway via PI3K/AKT. In this regard, however, little data has been collected.[102]

Curcumin, a hydrophobic polyphenol produced by Curcuma longa, has also attracted considerable interest because of its ability to interact and regulate multiple molecular targets. In particular, curcumin has been shown to inhibit the mTOR pathway and the phosphorylation of S6K, resulting in the inhibition of proliferation and in the induction of autophagy.[103] In addition, it can also activate Nrf2.[104]

3.3 Pharmacological approaches

The possibility to identify an anti-ageing or, better still, a pro-longevity drug is one of the main goals of pharmaceutical companies.[105] Because NSPs constitute the most interesting target for the achievement of healthy ageing, much research has been conducted with the aim to pharmacologically modulate insulin/IGF-1, mTOR, and sirtuin pathways.

Concerning the aim to decrease blood IGF-1 levels, a number of available drugs have been designed for human use. These can block IGF-1 signalling pathway, targeting cells and tissues involved in the production of, or in response to, GH and IGF-1 signals. In particular, somatostatin analogues control serum IGF-1 levels through the suppression of GH secretion by pituitary releasing factor. However, they also suppress the secretion of other endocrine hormones. On the

contrary, pegvisomant, an antagonist of the GH receptor, used for acromegaly treatment, might have positive effects on healthy ageing, lowering serum IGF-1, and increasing insulin sensitivity. Only a few serious adverse effects have been reported concerning the use of pegvisomant. Nevertheless, the effect of these drugs on health and lifespan is currently being investigated in several different experimental models.[4,27]

Rapamycin (or Sirolimus) is the most well-known TOR inhibitor. It was isolated from *S. hygroscopicus* and shows anti-proliferative and immunosuppressive activity. In female mice, it has shown to extend lifespan up to 30% at high doses and to a lesser extent in males.[106] This molecule allosterically inhibits mTORC1 kinase activity by the interaction with the mTOR carboxyl-terminus.[107] It doesn't binds mTORC2, so it selectively blocks only mTORC1.[108] Trials testing rapamycin in healthy elderly people are ongoing, hence, in a few years, it will be possible to determine its possible role as an anti-ageing drug.[2]

Sirtuin pathways can be triggered using NAD^+ precursors to increase NAD^+ levels, by activating NAD^+ biosynthetic enzymes or inhibiting NAD^+ hydrolase. In fact, the enzymatic reaction, catalysed by sirtuins, requires NAD^+ as a substrate, which is converted to nicotinamide. *In vivo*, nicotinamide acts as an inhibitor of the reaction, and provides negative feedback for the sirtuins.[71] The synthetic isonicotinamide can activate sirtuins by competing with its endogenous inhibitors. Unfortunately, its action depends on specific concentrations within the cell that limit the possibility to use this approach.[2,109] It is noteworthy that a precursor to NAD^+, called nicotinamide riboside, can be transformed by the organism into NAD^+ and put to use. Using this precursor, a supplement was created.

Metformin is one of the most widely used anti-diabetic agents but it was also identified as a possible anti-ageing drug, targeting a number of age-related mechanisms. It is a safe oral anti-hyperglycemic drug, belonging to the biguanide family that decreases blood glucose concentration, using different mechanisms to insulin. It acts by enhancing insulin sensitivity, inducing greater peripheral uptake of glucose, and decreasing hepatic glucose output. Nevertheless, although these

aspects are relevant for diabetes treatment, the same are not pertinent with ageing.[110] In addition, it acts specifically on NSPs involved in ageing process, decreasing insulin levels and IGF-1 signalling, inhibiting mTOR pathway, and activating AMPK energy sensor, hence positively affecting inflammation and autophagy.[111-114]. Other anti-ageing effects seemingly unrelated ~~to~~ NSPs have been described.[110] So, there is growing evidence that metformin increases healthy lifespan in model animals and epidemiological retrospective studies suggest that chronic treatment with metformin is associated with a decreased cancer incidence and with a decreased mortality rates. It is not surprising that a big study has been planned to elucidate the effect of metformin on ageing process, by measuring a composite outcome that includes cancer, dementia, cardiovascular diseases and mortality.[110]

Moreover, in cells treated with LPS, metformin has been shown to inhibit SASP through NF-κB inhibition. In particular, metformin was demonstrated to block NF-κB translocation to nucleus, and to inhibit the phosphorylation of some factors of the pathway.[115] In a model of high-fat-diet induced obesity mice, it has been suggested that the modulation of microbiota (through an increase of *Akkermansia (A.) muciniphila* population) by metformin may contribute to its antidiabetic effects.[116,117] In a cross-sectional study of the microbiota in women with T2DM, metformin treatment was accompanied by a marked increase in the bacterium *A. muciniphila* and an associated increase in mucin-producing goblet cells.[118] It is noteworthy that *A. muciniphila* adheres to the intestinal epithelium and strengthens enterocyte monolayer integrity in vitro, suggesting an ability to fortify an impaired gut barrier.[119]

4. Conclusion

Age-associated diseases are the biggest challenges in both developed and developing countries. In fact, the overall increase in average life expectancy is far greater than that of healthy life expectancy, ~~as~~ evidenced by their incremental burden of them. This is already overwhelming the healthcare and welfare systems of developed nations, and if present trends

continue, the challenges could cause even larger problems. A long life in a healthy, vigorous, youthful body has always been one of humanity's greatest dreams. Recent progress in genetic manipulations and calorie-restricted diets in laboratory animals hold forth the promises that someday science should enable us to exert total control over our own biological ageing. In particular, up regulation of some NSPs and down regulation of others ~~ones~~ are associated with age-related diseases, whereas their manipulation, in model animals, has been associated with extension of maximum lifespan and longevity.

At present, there is a scarcity of data on the efficacy of pharmacological treatment, whereas a dietetic approach seems to be feasible as a prevention for many age-related diseases.

5. Expert opinion

Our approach is directly to show possible interventions on NSPs, involved in ageing processes, to delay ageing and to get older more healthily. ~~Because ageing is an~~ ineluctable process, the strategies to live lon~~ger~~ in a healthy condition have been the main goal of recent researches.

Nutrition ~~n~~ represents the most concrete way to act on ageing. Accordingly, several kinds of dietary interventions, effective in model animals, have been outlined. However, they are not easily applicable to human beings. So, alternative approaches should be pursued. A feasible diet that reduces the risk of ageing-related diseases, favouring health ageing and longevity, should down regulate NSPs. The Insulin/IGF-1 pathway is activated by refined sugars, the mTOR pathway by amino acids and that of sirtuins, by nutraceuticals such as resveratrol. So, a diet with low GI, with healthier vegetable proteins, and rich in nutraceuticals, is to be recommended. However, it is not necessary to determine the ideal composition of this diet, because it does already exist in the traditional "poor" MedDiet.

The low content of animal protein and the low GI of the MedDiet modulate the insulin/IGF-1 and the mTOR pathways, with a down-regulation of the signals that lead to the inhibition of

FOXO, thus favouring the transcription of homeostatic genes involved in survival and longevity. The down-regulation of both IGF-1 and mTORC1 also induces an anti-inflammatory effect. In addition, nutraceuticals from vegetables act both as hormetins and sirtuin activators (i.e. reseveratrol).

In addition, Okinawan centenarians follow a vegetable-rich, low GI, and low-calorie diet, rich in nutraceuticals, with low assumption of meat, dairy products, and fat intake, and with the moderate intake of fish products. Compared to Western diets, this diet does not over stimulate mTOR, insulin, and IGF-1 receptors.

With regards pharmaceutical interventions, some drugs appear promising but further clinical studies are mandatory. We are ready both to consider several therapeutic options for the treatment of age-related diseases and to initiate clinical trials with the goal of increasing the healthy lifespan of human beings.

Rapamycin and somatostatins are already in use for other clinical indication, however they have notable side effects, which limit their consideration as an anti-ageing therapy. Moreover, the more safe safer pegvisomant has not yet been tested in clinical trials for healthy ageing, and neither has nicotinamide riboside.

The antidiabetic drug metformin appears to target a number of ageing-related mechanisms. In fact, it modulates the biology of ageing and health span in model organisms. It has been used with an excellent safety record for over 60 years, so it is the ideal drug to test in clinical trials to further understand its effect on healthy ageing and longevity. The trials are ongoing and hopefully in a few years the results will be available.

Article highlights box

- The global aged population is growing and the achievement of healthy ageing is the main challenge of the recent research. So, the identification of new therapeutic targets, supporting healthy lifespan, constitutes the main goal of ageing studies.

- NSPs like insulin/IGF-1, mTOR and sirtuins, play a central role in the modulation of lifespan in various organisms. Their modulation influences ageing and longevity processes.
- At present, dietary interventions represent the only possibility to modulate ageing, with a non-invasive method.
- Nutraceuticals have already been identified as possible molecules with bioactive effects on ageing process.
- Drugs or supplements may hold the key to the future creation of a longevity elixir.

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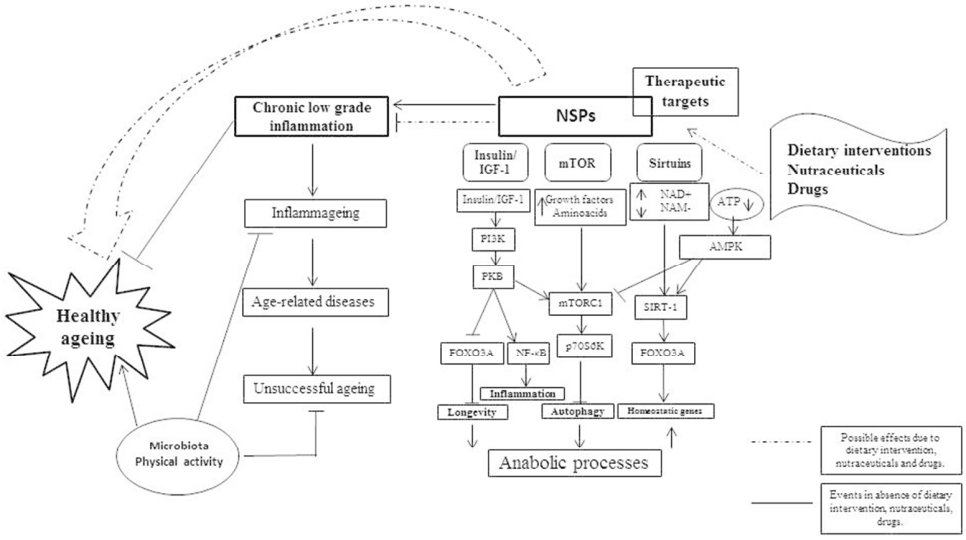
Legend to Figure

Figure 1. Overview on NSPs and their role in healthy ageing.
The figure shows molecular events involved in insulin-IGF-1, mTOR and sirtuin pathways, known as NSPs, and the possible effects due to their modulation by dietary interventions, nutraceuticals, and drugs. These act inhibiting insulin/IGF-1 and mTOR pathways and increasing that of sirtuins, ~~fav~~ouring longevity, autophagy and the activation of the homeostatic genes, and reducing inflammation and anabolic processes. Microbiota and physical activity are external factors that influence successful ageing, through inflammation control.
See the text for the acronyms.



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VI. Nutraceutical effects of table green olives: a pilot study with *Nocellara del Belice* olives

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RESEARCH Open Access

Nutraceutical effects of table green olives: a pilot study with *Nocellara del Belice* olives

Giulia Accardi[1*†], Anna Aiello[1*†], Valeria Gargano[2], Caterina Maria Gambino[1], Santo Caracappa[2], Sandra Marineo[2], Gesualdo Vesco[2], Ciriaco Carru[3], Angelo Zinellu[3], Maurizio Zarcone[4], Calogero Caruso[1] and Giuseppina Candore[1]

Abstract

Background: The aim of this study was to analyse the nutraceutical properties of table green olives *Nocellara del Belice*, a traditional Mediterranean food. The Mediterranean Diet has as key elements olives and extra virgin olive oil, common to all Mediterranean countries. Olive oil is the main source of fat and can modulate oxidative stress and inflammation, whereas little is known about the role of olives. Moreover, emerging evidences underline the association between gut microbiota and food as the basis of many phenomena that affect health and delay or avoid the onset of some age-related chronic diseases.

Methods: In order to show if table green olives have nutraceutical properties and/or probiotic effect, we performed a nutritional intervention, administering to 25 healthy subjects (mean age 38,3), 12 table green olives/day for 30 days. We carried out anthropometric, biochemical, oxidative stress and cytokines analyses at the beginning of the study and at the end. Moreover, we also collected fecal samples to investigate about the possible variation of concentration of *Lactobacilli*, after the olives consumption.

Result: Our results showed a significant variation of one molecule related to oxidative stress, malondialdehyde, confirming that *Nocellara del Belice* green olives could have an anti-oxidant effect. In addition, the level of interleukin-6 decreased significantly, demonstrating how this food could be able to modulate the inflammatory response. Moreover, it is noteworthy the reduction of fat mass with an increase of muscle mass, suggesting a possible effect on long time assumption of table olives on body mass variation. No statistically significant differences were observed in the amount of *Lactobacilli*, although a trend towards an increased concentration of them at the end of the intervention could be related to the nutraceutical effects of olives.

Conclusion: These preliminary results suggest a possible nutraceutical effect of daily consumption of green table olives *Nocellara del Belice*. To best of our knowledge, this is the first study performed to assess nutraceutical properties of this food. Of course, it is necessary to verify the data in a larger sample of individuals to confirm their role as nutraceuticals.

Keywords: Table green olives, Mediterranean Diet, Nutraceuticals, Dietary intervention, Oxidative stress, Inflammatory status

Background

Nowadays, ageing process and the related diseases constitute one of the bigger challenges in Western countries. The general increase of lifespan does not go, hand in hand, with the increase of healthy lifespan, the so-called "healthspan". This constitutes a worldwide problem, in particular due to age-related chronic diseases [1].

It is well known that the pathogenesis of age-related diseases is characterized by a low-grade inflammation. In particular, the visceral adipose tissue is a source of inflammatory mediators produced by adipocytes and infiltrating monocytes [2].

Abdominal obesity with dyslipidaemia, elevated blood pressure and impaired glucose tolerance characterizes metabolic syndrome (MS) that predisposes to the onset of age-related diseases. As many studies demonstrate, a

* Correspondence: giuliabio@gmail.com; anna.aiello2903@gmail.com
†Equal contributors
[1]Sezione di Patologia generale del Dipartimento di Biopatologia e Biotecnologie Mediche (DIBIMED), Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy
Full list of author information is available at the end of the article

dietary Mediterranean regimen can positively influence these parameters. Large intervention trials showed, in fact, that Mediterranean Diet (MedDiet) could prevent and or delay the onset of age-related diseases with a great implication in the health social system [3–7].

The traditional MedDiet is a common dietary pattern, adopted by inhabitants of countries within Mediterranean basin where the olive tree, *Olea europaea*, is widely cultivated for the production of table olives and oil. They are the essential components of the MedDiet with a very significant economic value. Besides of the economical contribution to national economies, these are important in terms of nutritional value. Extra virgin olive oil (EVOO) has been claimed to play a key role in the prevention of age-related diseases and in the attainment of longevity. This is due to the high levels of monounsaturated fatty acids, likely responsible for the decreased low density lipoprotein levels, and phenolic compounds claimed to play a role as antioxidants and anti-inflammatories [6, 8, 9].

Foods with bioactive molecules can be considered "nutraceuticals", defined as "Naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products, and have health promoting, disease preventing, or medicinal properties". The term was coined in 1989 by Stephen De Felice and was born from the conjunction between nutrition and pharmaceutics [10].

As reported in a recent review, table olives are extremely rich sources of polyphenols, especially oleuropein and hydroxytyrosol, comprising 1–3 % of the fresh pulp weight. Despite the high levels of hydroxytyrosol in both table olives and EVOO, in humans its bioavailability was proved only in oil. Accordingly, to the best of our knowledge, there are no human studies on health effects of table olives [11]. However, the amount of polyphenols is strongly influenced by the variety and the geographical origin. *Greek Koroneiki* have a very high level of them, while the polypenol content of the *Spanish Arbequina* is low and that of *Sicilian Nocellara* is medium-high [12]. So, a possible anti-inflammatory and anti-oxidant effect of these Sicilian olives is conceivable.

The development of strategies aimed at counterbalancing the frailty in the elderly is a major challenge for the medicine of 21st century [1]. As recently reviewed, ageing affects the gut microbiota composition and its influence in immune response. Age-related gut microbiota changes are associated with immunosenescence and inflamm-ageing. Hence, the gut ecosystem shows the potential to become a promising target for strategies able to contribute to the health status of elderly. In this context, the consumption of pro/prebiotics may be useful in both prevention and treatment of age-related pathophysiological conditions, favouring the attainment of longevity [13].

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". *Lactobacilli* (*L.*) and *Bifidobacteria* are the most commonly used bacterial probiotics [14]. Nutritional supplementation in aged people might help to maintain good immune-inflammatory responses by re-equilibrating the gut microbiota.

Fermentation is one of the oldest methods to preserve olives. It has applied worldwide for thousands of years. The microbiota of olives during fermentation, that varies somewhat from *cultivar* to *cultivar*, has been recently reviewed, showing that *L.* are the major constituents of *Nocellara del Belice* olives microbiota [15, 16]. So, a possible probiotic-like effect of these olives is feasible.

The aim of this pilot study was to evaluate the effect of green table olives *Nocellara del Belice* on clinical and biological parameters of healthy individuals at baseline (T0) and after the assumption of 12 olives/day for 30 days (T1) (this amount was chosen to assure the administration of $2x10^7$ *L.*/die, see below).

Results and discussion

Hematochemical tests

At the end of the intervention, all hematochemical parameters did not experienced variations, with the exception of alkaline phosphatase that significantly increased (Table 1).

However, the increased values were in normal range. This means that a regular consumption of 12 green olives/day for 30 days does not have a detrimental effect on liver and kidney function and on lipid values.

Anthropometric measurements

At T1, in analysed subjects the fat mass significantly decreased together to an increase of muscle mass (Table 1). The possible explanation could be linked to the capacity of conjugated linoleic acid (CLA) to reduce the body fat levels [17]. This molecule is present both in EVOO and

Table 1 The Table shows the arithmetic average values at T0 and T1, the p-value and the variation in percentage (+ indicates an increase of the variable at T1; - a decrease at T1)

Variable	T0 ± SD	T1 ± SD	p-value	%
Alkaline phosphate (IU/L)	49.95 ± 13.26	53.73 ± 16.81	0.022	+7.57
Fat mass%	29.70 ± 7.92	28 ± 7.24	0.004	−5.72
Muscle mass %	66.97 ± 7.62	68.36 ± 6.85	0.003	+2.09
IL-6 (FI)	31.52 ± 29.37	20.89 ± 11.93	0.027	−33.73
MDA (μmol/L)	2.72 ± 0.64	2.33 ± 0.49	0.005	−14.24
Weight (Kg)	70.44 ± 14.07	69.93 ± 13.77	0.08	−0.72
BMI (Kg/m^2)	24.37 ± 4.19	24.24 ± 4.16	0.22	−0.53

T0 baseline, *T1* the end of the nutritional intervention (30 days), *BMI* Body Mass Index, *IL-6* interleukin-6, *IF* indirect fluorescence, *MDA* malondialdehyde, *SD* standard deviation

table olives, and can also be produced during their digestion. In experimental models, acting as signalling mediators, CLAs inhibit lipogenesis, increase fat oxidation, and reduce adipocytes size [18, 19].

Cytokines analyses

The serological analysis of the levels of the main pro and anti-inflammatory cytokines was conducted. Although it was not possible to evaluate the absolute concentration of interleukin (IL)-6 because it is too low, a significant variation was measured in the indirect fluorescence (IF). In fact, its levels significantly decreased at the end of the dietary intervention (Table 1).

IL-6 is a pleiotropic cytokine capable of regulating proliferation, differentiation and activity in a variety of cell types. In particular, it plays a pivotal role in acute phase responses and in the balancing of the pro and anti-inflammatory pathways. It is involved in impaired lipid metabolism and in the production of triglycerides. Moreover, it decreases lipoprotein lipase activity and monomeric lipoprotein lipase levels in plasma which contributes to increased macrophage uptake of lipids [20]. This datum suggests that a regular consumption of green olives can have anti-inflammatory effects linked to polyphenols, known to have anti-inflammatory properties [6].

Oxidative stress analyses

At the end of intervention, the values of malondialdehyde (MDA) significantly decreased (Table 1), while paraoxonase (PON) plasma levels and reduced glutathione in the red blood cells were not changed (data not shown). MDA is the main product of the polyunsaturated fatty acids peroxidation and is an important index of oxidative stress [21]. So, its reduction should be linked to the increased assumption of mono-unsatured oleic acid by olives.

Microbiological analyses on feces

The amount of *L.*/g of feces was quantified before and after the intervention. No statistically significant differences were observed, although a trend towards an increased amount of *L.* was highlighted in some subjects at T1 (data not shown). Thus, we can speculate that a more durable dietary intervention and a bigger sample of people could give more interesting results.

Conclusions

The traditional MedDiet is a common dietary pattern that identify a lifestyle and a culture. It was proven that it contributes to better health and quality of life. Concerning its healthy effects, low content of animal protein and low glycaemic index may directly modulate the insulin/insulin-like growth factor-1 and the mammalian target of rapamycin pathways, known to be involved in ageing, age-related diseases and longevity. In addition to the influence on nutrient sensing pathways, many single components of MedDiet are known to have positive effects on health, reducing inflammation, oxidative stress and other important risk factors of age-related diseases [6].

This pilot study demonstrates an anti-inflammatory and anti-oxidant effect of daily consumption of green table olives *Nocellara del Belice*. Moreover, it is noteworthy the reduction of fat mass with an increase of muscle mass. Although no statistically significant probiotic effect was observed, the positive trend related to *L.* amount at T1 could represent a starting point for further studies.

It is to note that the study presents limitations. One is strictly related to the intrinsic complexity of human as study model and to the inter/intra-individual variability. These features are more evident in ageing than in younger people. This is the reason why we chose middle age people. So, our choice represented the second limitation of the study because we did not analyze the effects of the intervention in elderly. Thirdly, it is necessary to verify these data in a larger sample of individuals to confirm the role of table green olives as nutraceutical foods. Also the duration of the intervention could be inadequate. In fact, we developed a short-term dietary regimen (30 days). This is a good choice in terms of compliance to the study because the more is the time of intervention the more is the drop out effect. But, a long-term dietary intervention could be stronger in terms of variation of analyzed parameters (e.g., *L.* amount in feces).

However, these new knowledges give an important achievement for the food and farming industry, especially in Sicily, where the olives represent a great potential resource. No approved healthy property and claim exist for them. Therefore, adding such a common product to the class of nutraceuticals could represent a big deal.

In the era of many expansive and mysterious longevity elixirs, the olives could represent a traditional, cheap and accessible to everyone “healthy food”.

Methods

Study design

The trial consisted in the assumption of 12 olives/day for 30 days. They belonging to the variety *Nocellara del Belice*, were processed in salt solution without any chemical additives.

See Fig. 1 for the flow chart of the study design.

Study population

Twenty-five randomized volunteers (mean age 38,3), both men and women, were recruited from April 2015 to July 2015. The subjects included were: healthy, with age between 18 and 65 years and Caucasian. The

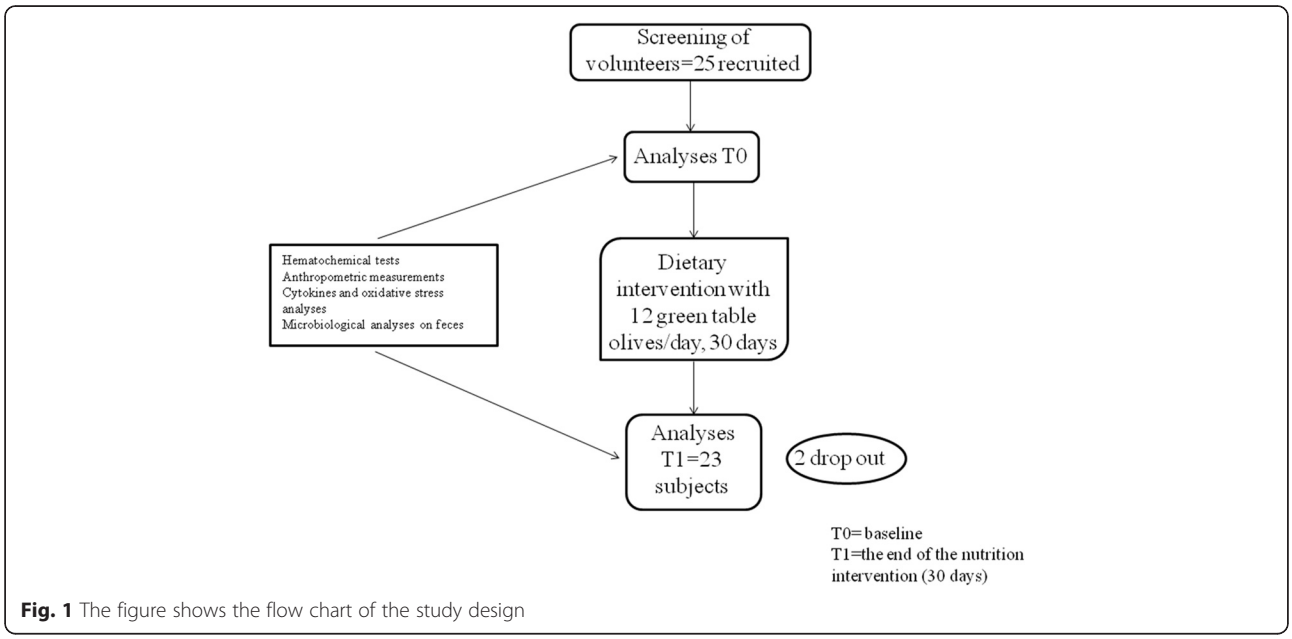

Fig. 1 The figure shows the flow chart of the study design

exclusion criteria provided: a history of the absence of pathologies (obesity, MS); a history of use of any pre or probiotics as dietary supplements within 3 months prior to the study; a history of treatment with statins or similar and with lyposoluble drugs; the onset of gastrointestinal disorders or the use of antibiotics during the nutritional intervention. No restriction related to sex was considered. Two subjects dropped out of the trial. All participants signed an informed consent before the enrolment. To respect the privacy, everyone was identified with an alphanumeric code. Height and weight were measured wearing light clothes and barefoot. The body composition was registered using specific hardware and software. Body mass index was calculated as weight (in kilos) over height squared (in square metre) (Table 1). Dietary habits were assessed through a food frequency questionnaire, officially validate by the EPIC study. Blood tests, oxidative stress and cytokines analyses were carried out for all subjects at T0 and T1. Molecular analyses were conducted on *L.* DNA obtained from fecal samples to measure the variation of its amount. A database was created to insert all participants' data and to handle the collected information.

Hematochemical tests

The recruited people underwent to venipuncture at T0 and at T1. Blood samples were collected in specific blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) for plasma analyses and in serum tubes with no additives. Plasma and sera were separated from whole blood by low-speed centrifugation at 2,500 rpm for 15′ at 4 °C. After separation, the samples were stored at −80 °C for further tests. We also obtained gruel of red blood cells from blood collected in tubes with EDTA through three washes with physiological solution (centrifugation at 2,500 rpm for 15′ at 4 °C).

Evaluation of parameters of oxidative stress

These analyses were conducted in collaboration with the University of Sassari. Thiobarbituric acid reactive substances (TBARS) were determined according to the method described by Esterbauer and Cheeseman [22]. TBARS methodology measures MDA and other aldehydes produced by lipid peroxidation induced by hydroxyl free radicals. For the measurements, plasma was mixed with 10 % trichloroacetic acid and 0.67 % thiobarbituric acid and heated at 95 °C in thermoblock heater for 25′. TBARS were determined by measuring the absorbance at 535 nm. A calibration curve was obtained using standard MDA and each curve point was subjected to the same treatment as that of the samples.

PON activity was determined by measuring the increase in absorbance at 412 nm (formation of 4-nitrophenol), using paraoxon (O, O diethyl-O-p-nitrophenyl phosphate) as a substrate [22]. The enzyme activity was calculated by using the molar extinction coefficient of 17,100 $M^{-1}cm^{-1}$ and one unit of PON activity was defined as 1 nanomole of 4-nitrophenol formed per minute. For red blood cell glutathione quantification, 200 μl (μL) of thawed packed cells were lysed by adding 600 μL of cold water and keeping the samples at 4 °C for 15′. 200 μL of lysed samples were deproteinized by adding 200 μL acetonitrile and centrifuged at 2,000 × g for 5′. Samples were then derivatized by mixing 100 μL of supernatant with 100 μL of sodium phosphate buffer (60 μmol (mmol)/L, pH 12.5), and 25 μL of 5-

Iodoacetamidofluorescein (4.1 mmol/L). After vortex mixing, samples were incubated for 15′ at room temperature. Derivatized samples were diluted 100-fold in water and analysed by capillary electrophoresis with laser induced fluorescence detection [23].

Pro and anti-inflammatory cytokines analyses

These analyses were conducted using Luminex assays, coupled to Bio-Plex Manager software.

Data obtained have been checked by technical department and quality control parameters. Values of the standard curve were compared to the values provided by the manufacturer of the kits used and must not exceed a CV of 15 %. All of above parameters were applied on, at least, the 90 % of the standard curve values.

Microbiological and molecular analyses of *Lactobacilli*

In order to quantify the amount of *L.* in each olive, 1 g of pulp was suspended in phosphate-buffered saline solution (1 mL), homogenized for 2′ at maximum speed, and then serially diluted. Decimal dilutions were plated and incubated on *de Man, Rogosa and Sharp*e at 30 °C for 48 h to observe the *L.* growth. The colonies' count was performed in triplicate and the *L.* DNA was extracted from them to perform molecular analyses. Moreover, colony suspension were used as a template for Real Time PCR. The primers and probes used to detect *L. species* (*spp*) were based on 16S rRNA gene sequences retrieved from the NCBI databases (Table 2). The amplification reactions were carried out in a total volume of 25 ml containing 1X SSoFast Probe mix (BIORAD), primers (each at 200 nM concentration), 100 nM TaqMan MGB probe, 60 ng purified target DNA. Amplification (1 cycle of 5′ at 95 °C, 45 cycles of 15′′ at 95 °C and 1 cycle of 1′ at 60 °C) and detection were carried out on a CFX Real Time system (BIORAD).

Fluorescent probe was labeled at 5′ end with the reporter dye 6-carboxyfluorescein and at 3′ end with a quencher dye. A negative and a positive control were included on the reaction plate.

In order to perform the quantification of *L.* in each feces sample, the QIAamp DNA Stool Minikit (Qiagen) was used to extract DNA from an appropriate amount of frozen stool sample, according to the manufacturer's instructions. The Real Time PCR was performed as previous described and the cycle threshold of each sample was compared to a standard curve made by diluting genomic DNA (10-fold serial dilution) from cultures of known concentrations of *L.* (10^6 CFU/ml).

Table 2 Primer and probe sequences for analyses of *L. spp* in Real Time PCR

	Oligonucleotide sequence 5′-3′
Primer Forward	GAGGCAGCAGTAGGGAATCTTC
Primer Reverse	GGCCAGTTACTACCTCTATCCTTCTTC
Probe	FAM-ATGGAGCAACGCCGC-QUENCER

Statistical analyses

The paired comparisons were performed with the Student's *t*-test or the Wilcoxon signed rank test, according to the normality of samples. Statistical analyses were performed with the IDE RStudio for the R (version 3.2.2) software [24, 25].

Competing interests

The authors declare that they have no competing interest.

Authors' contribution

GA, AA, VG, and CC* conceived and designed the study; GA, AA, CMG, VG, SM, SC, GV, AZ, CC* and CC** performed or supervised experiments. MZ executed statistical analysis. GA, AA, VG, CC*, and CC** analyzed and interpreted data; GA and AA drafted the paper. GC, CC* and CC** made critical revisions to the draft. All authors read and approved the final manuscript. CC*: Calogero Caruso. CC**: Ciriaco Carru

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Author details

[1]Sezione di Patologia generale del Dipartimento di Biopatologia e Biotecnologie Mediche (DIBIMED), Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy. [2]Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, 90129 Palermo, Italy. [3]Dipartimento di Scienze Biomediche, Università di Sassari, Viale San Pietro 43/b, 07100 Sassari, Italy. [4]UOC Epidemiologia Clinica con registro tumori di Palermo e provincia, AOUP "Paolo Giaccone", Palermo, c/o Dipartimento di Scienze per la promozione della salute e materno infantile "G. D'Alessandro", Università di Palermo, Via del Vespro 133, 90131 Palermo, Italy.

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VII. β-Glucans and Post Prandial Satiety: the Role of Intestinal Hormones in Healthy Volunteers (*submitted*)

β-Glucans and Post Prandial Satiety: the Role of Intestinal Hormones in Healthy Volunteers

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Complete List of Authors:	Baldassano, Sara; Universita degli Studi di Palermo, STEBICEF Buscemi, Silvio; Universita degli Studi di Palermo, DIBIMIS Aiello, Anna; Universita degli Studi di Palermo, DIBIMEF Accardi, Giulia; Universita degli Studi di Palermo, DIBIMEF caruso, calogero; Universita degli Studi di Palermo, DIBIMEF vasto, sonya; STEBICEF;

Keywords:	β-glucans, entero-endocrine system, intestinal hormones, microbiota, nutritional intervention

Abstract:	Recent interest in intestinal hormones has risen with the idea that they modulate glucose tolerance and food intake through a variety of mechanisms, and such hormones like peptide YY (PYY), ghrelin, glucagon-like peptide (GLP)-1 and 2, and cholecystokinin (CKK) are therefore excellent therapeutic candidates for the treatment of diabetes and obesity. Furthermore, in the recent years, multiple studies suggest that the microbiota is critically important for normal host functions, while impaired host microbiota interactions contribute to the pathogenesis of numerous common metabolic disorders. In this study, we considered the nutraceutical effects of β-glucans added to pasta at the concentration of 6g\100g. Ten participants have been recruited and hematochemical analyses and intestinal hormones tests have been performed before and after 30 days of pasta intake. Stool specimens have been studied for Lactobacillus Fermentum, Lactobacillus acidophilus, Lactobacillus salivarius, Bifidobacterium longum, and Enterococcus faecium presence before and after 30 days of nutritional intervention. After 30 days of regular intake of pasta enriched by β-glucans results have been evaluated. In conclusion, pasta prepared from barley flour enriched with β-glucans at 6% exhibit promising responses on glucose metabolism, on intestinal hormones responses and on microbiota modification.

β-Glucans and Post Prandial Satiety: the Role of Intestinal Hormones in Healthy Volunteers

Sara Baldassano[1], Silvio Buscemi[2], Anna Aiello[3], Giulia Accardi[3], Calogero Caruso[3], Sonya Vasto[1,4]*

[1] Department of Biological Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze Parco d'Orleans, Edificio 16, 90128, Palermo, Italy; sonya.vasto@unipa.it; sara.baldassano@unipa.it

[2] Biomedic Department of Internal and Specialistic Medicine (DIBIMIS), University of Palermo, Via del Vespro 129, 90127 Palermo, Italy; silvio.buscemi@unipa.it

[3] Department of Pathobiology and Medical Biotechnologies (DIBIMED), University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; calogero.caruso@unipa.it; giuliabio@gmail.com; anna.aiello2903@gmail.com

[4] Institute of biomedicine and molecular immunology "Alberto Monroy" CNR, Via Ugo la Malfa, 153, 90146 Palermo, Italy; sonya.vasto@unipa.it

* Correspondence: sonya.vasto@unipa.it; Tel.: +39-09123897508

1.Summary: Recent interest in intestinal hormones has risen with the idea that they modulate glucose tolerance and food intake through a variety of mechanisms, and such hormones like peptide YY (PYY), ghrelin, glucagon-like peptide (GLP)-1 and 2, and cholecystokinin (CKK) are therefore excellent therapeutic candidates for the treatment of diabetes and obesity. Furthermore, in the recent years, multiple studies suggest that the microbiota is critically important for normal host functions, while impaired host microbiota interactions contribute to the pathogenesis of numerous common metabolic disorders. In this study, we considered the nutraceutical effects of β-glucans added to pasta at the concentration of 6g\100g. Ten participants have been recruited and hematochemical analyses and intestinal hormones tests have been performed before and after 30 days of pasta intake. Stool specimens have been studied for Lactobacillus Fermentum, Lactobacillus acidophilus, Lactobacillus salivarius, Bifidobacterium longum, and Enterococcus faecium presence before and after 30 days of nutritional intervention. After 30 days of regular intake of pasta enriched by β-glucans results have been evaluated. In conclusion, pasta prepared from barley flour enriched with β-glucans at 6% exhibit promising responses on glucose metabolism, on intestinal hormones responses and on microbiota modification.

Key words: β-glucans; entero-endocrine system; intestinal hormones; microbiota; nutritional intervention

2. Introduction

The β-glucans are soluble fibres commonly found in the cell wall of yeasts, fungi, bacteria, algae and cereals. All the β-glucans are polysaccharides consisting of linear molecules of D-glucose joined together by glycosidic bonds linear β (1-3) and β (1-4) and differ between them for the length and branched structures. The branches derived from the nuclear chain glycoside are highly variable and the two main groups are branching chains glycosidic β (1-4) and β (1-6). These ramifications appear to be specific. For example, the β-glucans of mushrooms have side branches 1→6 while those of bacteria have side branches 1→4 [1, 2]. The presence of the bond β (1-3) leads to the formation of folds in the linear chain that allow water to enter; for this reason the β-glucans are classified as soluble fibres. Characteristics of β-glucans are their effect on cholesterol that depends on the ability to form a viscous layer on the surface of the small intestine that reduces the absorption of cholesterol and the bile acids. The inhibition of the reabsorption of bile acids can increase their synthesis from endogenous cholesterol thus reducing the blood levels of low density lipoprotein (LDL)-cholesterol [3]. This action has already been investigated using breakfast drinks [4], biscuits and crackers added with less of 3% of β-glucans [3]. A minimum dose of 3g/day has been demonstrated to reduce the blood levels of cholesterol and consequently it decreases the risk of cardiovascular diseases [4]. In fact, the β-glucans viscous layer may have beneficial effects on glycaemia and sensations of hunger and satiety [4,5]. In a recent report, some of us considered the nutraceutical effects of β-glucans added to pasta (6%). After 30 days of pasta intake, we obtained encouraging results with a significant decrease of LDL-cholesterol, interleukin (IL)-6 and advanced glycation end-product levels [6]. Therefore, the nutraceutical use of β-glucans seems to be an interesting perspective [7].

On the other hand, the effects of β-glucans on satiety have been reported in many studies, but no consensus has been reached. In a recent report, it was examined the effects of breakfasts varying in the dose of oat bran (4g or 8g β-glucans). The sensations associated with hunger and satiety were evaluated using visual analogue scales before and after ingesting the test breakfasts and every 30 min until 210 min. Oat bran addition in breakfasts increased postprandial satiety [8]. However, the effects of regular intake in human of higher amounts of β-glucans on intestinal-hormones, that concur to regulate appetite/satiety, have not been defined.

Moreover, many studies demonstrated the role of gut microbiota in many inflammatory diseases, including type 2 diabetes mellitus and obesity. Two groups of beneficial bacteria are dominant in the human gut, the Bacteroidetes and Firmicutes. The relative proportion of Bacteroidetes is decreased in obese people by comparison with lean people, and this proportion

increases with weight loss, indicating that obesity has a microbial component [9]. Thus, the alteration of microbiota homeostasis can be involved in the pathogenesis of diseases but it is not clear if this process is a consequence or a cause of it. In animal model modification of intestinal microbiota seems to affect insulin resistance and post prandial glucose response perhaps due to reduced gut permeability and therefore influencing intestinal hormones concentration. More interestingly, mice model treated with prebiotics showed increased levels some intestinal-hormones [10].

Microbiota has been already identified as a metabolic organ that could ferment non digestible dietary components. In fact, microbiota generates short chain fatty acids (SCFAs), which could affect host energy metabolism and could develop obesity by changing the hormonal secretion in the intestine. So, microbiota cyclical fluctuation in composition may trigger intestinal hormones release. In addition, in obese rats and high-fat diet-fed mice an intervention with prebiotics stimulates Bifidobacteria and reduces weight, by increasing intestinal-hormone PYY and reducing ghrelin [11]. In obese people during weight gain it is observed a decreased level of GLP-1, which is normally regulated by gut microbiota. GLP-1 influences the expression of the proglucagon (its precursor), which causes insulin resistance independent of fatty acids circulating level [12].

Thus, in the present pilot study we investigated the modification of serum concentrations of orexigenic peptide ghrelin, anorexigenic active form of GLP-1, GLP-2, CKK, and PYY. Those hormones were evaluated in the postprandial period after a challenge with a test meal constituted by pasta supplemented with 6% β-glucans.

This study was performed in overweight and healthy individuals, before and after 30 days of pasta intake. In addition, we quantified the variation of some bacterial strains in faeces samples at the baseline and after 30 days of nutritional intervention.

3. Material and Methods

3.1. Study Design

This was a 30±3 days longitudinal, intervention study on the effect of consuming 100 g of pasta supplemented with 6% of β-glucans four times a week, in 10 overweight young Sicilians, otherwise healthy. It was not recommended other changes in dietary habits. We took blood for hematochemical test at baseline (day 0), as a control, and after 30 days (day 30) to register possible variation. In addition, we evaluated intestinal hormones after a challenge with test meals at day 0 and at day 30; we also collected stool samples in sterile containers for the analysis of some bacterial species of the intestinal microbiota. Moreover, anthropometric measurement (height in cm, weight in kg) were collected and BMI (kg/m2) was calculated.

The study protocol was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the local review board. Suitable subjects were identified from computerized clinic registers, according to the inclusion and exclusion criteria and were contacted by the clinicians personally or by telephone. All eligible candidates had to provide signed informed consent before enrolment in the study.

3.2. Test Meal

The intestinal hormones evaluation consisted of 100g of pasta with β-glucans administered to the selected people after night fasting of day 0 and of day 30 as sort of breakfast. Immediately after test meal blood samples were collected after 15, 30, 60, 90 and 180 min. Intestinal hormones measured were the following GLP-1, ghrelin, PYY, CCK, and GLP-2 (Figure 1).

3.3. Subjects

Inclusion criteria were: age range 20-30 years, overweight (BMI, 25-29.9 kg/m^2;), normal glucose tolerance (fasting plasma glucose <100 mg/dL; glycated haemoglobin <5.7%), slight dyslipidemia (total cholesterol 200-240 mg/dL, LDL 130-160 mg/dL, triglyceride level ≤170 mg/dL). Exclusion criteria were: any diagnosis of systemic disorder (including heart disease, hypertension, rheumatologic disease, liver, kidney and enterological disorders), any drug treatment, eating behaviour disease based on the VAS Questionnaire [13], any hypocaloric dietary treatment in the last six months. Enrolled people underwent a complete medical evaluation by a trained physicians (SB). All the subjects ended the pilot study (100% of trial completion), none abandoned the study before completion.

Participants were asked to maintain their usual diet and physical activity level throughout the study period. Body weight, height and blood pressure of all participants were measured and blood samples and faeces were collected at day 0 and at day 30. Baseline and after 30 days tests were performed between 8:00 and 9:00 a.m., in post-absorptive fasting from at least 10 hours, without any strenuous physical activity in the last three days. An indwelling venous cannula was placed in the forearm 10 min before the first blood sample. Blood samples were collected before and 15, 30, 60, 90, 180 min after the ingestion of the test meal and immediately centrifuged to collect serum samples, and stored at -80°C. Furthermore, faeces were stored at -80°C for subsequent determinations .

3.4. Nutritional Intervention

β-glucans extraction, characterization and pasta have been prepared according to Montalbano et al [14] by pasta factory Tomasello (Casteldaccia, Italy).

3.5. Haematochemical Tests

Plasma and serum samples were used for hematochemical test, total cholesterol, LDL-cholesterol, HDL-cholesterol, alanine aminotransferase, aspartate aminotransferase, glucose, triglycerides, creatinine, gamma-glutamyl transferase, total protein, uric acid, high sensitivity c-reactive protein.

3.6. Intestinal Hormones Tests

For intestinal hormones measurement, plasma was obtained from peripheral blood sample by centrifugation at 5°C for 10 min at 3000 rpm; plasma samples were collected in prechilled EDTA-containing tubes with apoprotein (0.6 TIU/ml blood) and dipeptidyl peptidase IV inhibitor (10 µl/ml blood). All samples were immediately stored at -80°C until analyzed. Results were obtained from all the study participants (see flow chart Figure 1). Samples were analyzed using the following enzyme immunoassay kit: EGLP-35K for active GLP-1, EZGRT-89K for total ghrelin, EZHPPYYT66K for total PYY, EZGLP-237K for GLP-2, all from Millipore (EMD Millipore, MERCK KGaA, Darmstadt, Germany); CCK (26-33) for CCK from Phoenix (PHOENIX PHARMACEUTICALS, INC.) These kits are enzyme immunoassays developed for the detection and quantization of the different hormones in plasma, serum or other tissue culture methods. Each kit provides sufficient reagents to perform up to 96 assays including standard curve and unknown sample. The detection method is fluorescent and/or colorimetric.

3.7. Microbiota Analysis

In order to perform the quantification of microbiota in faeces samples at the baseline and after 30 days of nutritional intervention, the total DNA amount (bacterial and human) was extracted with the QIAamp DNA Stool Minikit (Qiagen), from a frozen stool sample of 200 mg, quantified by a spectrophotometric assay. The total DNA was used as a template to perform the PCR Real_Time to identify the possible variation in the amount of the following bacterial strains Lactobacillus fermentum, Lactobacillus acidophilus, Lactobacillus salivarius, Bifidobacterium longum, Enterococcus faecium, Clostridium difficile. We have chosen Lactobacilli and Bifidobacterium as markers of healthy status of gut because they are considered reference probiotics (15, 16). Instead, we have selected Clostridium difficile, either as marker of intestinal inflammation (17) either because Lactobaccilli inhibit its growth (18). Finally, Enterococcus

faecium was preferred as marker of dysbiosis in overweight subjects (19). The amplification reactions were carried out starting from 1 µl DNA, 25µg/µl and Qiagen kits were employed, according to the manufacturer's procedure. Each assay contained a negative (without DNA) and a positive control (quantified DNA, specific for each strain). Moreover, specific primers and fluorescent probe labelled with the reporter dye 6-carboxyfluorescein (FAM) were used. In particular, the kits have the following catalog number: BBID00188A for Lactobacillus fermentum; BBID00184A for Lactobacillus acidophilus; BBID00196A for Lactobacillus salivarius; BBID00067A for Bifidobacterium longum; BBID00143A for Enterococcus faecium; BBID00100A for Clostridium difficile. The variation of DNA amount before and after the nutritional intervention was calculated considering the cycle threshold (CT) of each sample.

3.8. Statistical Analysis

Results are shown as mean ± S.E.M. for intestinal hormones data, S.D. used for haematochemical tests. The comparison of hematochemical tests before and after the 30 days of pasta intake was performed by Wilcoxon test. The comparison between the levels of gut hormones after test meal at baseline and after 30 days was performed by ANOVA followed by Bonferroni's post-test test using Prism Version 6.0 Software (Graph Pad Software, Inc., San Diego, CA, USA). The AUC values were calculated using the trapezoidal rule. In the field of pharmacokinetics, the AUC is the area under the curve in a plot of concentration of drug in blood plasma against time. In this case, it determines whether the same standardized two formulations of test meal, before and after 30 days treatment within pasta added with β-glucans, release the same dose of hormones. A p value <0.05 was considered to be statistically significant.

4. Results

4.1. Hematochemical Tests

Anthropometric and biochemical measurements are presented in Table 1. In particular, biochemical values did not change following the 30 days intervention with β-glucans with the exception of glucose concentrations and total and LDL cholesterol, as reported in Table 1. Body weight and body mass index (BMI) were not significantly changed at the end of the study. Thus, as observed ex-vivo in the previous study [6], the treatment with β-glucans induced a reduction of serum LDL-cholesterol concentrations.

Instead, in this study there were no significant alterations of high density lipoprotein (HDL)-cholesterol and triglycerides concentrations or the other considered parameters, probably, due to the small sample size.

On the other hand, we have observed a significant reduction of glycaemic levels after the treatment, datum not observed in the previous study, likely because previously only healthy normal weight subjects were studied.

4.2. Gut Hormone Responses

GLP-1 (Figure 2). The consumption of test meal induced an increase in plasma GLP-1 concentrations, starting from 15 min up to 90 min, peaking at 30 min. The consumption of the β-glucans enriched pasta for 30 days significantly increased the levels of GLP-1 at 0, 30 and 60 minutes after intake. As shown by area under the curve (AUC), on the whole after 30 days treatment we observed an increased release of GLP-1.

Ghrelin (Figure 3). Plasma ghrelin concentrations were higher in fasting state and they were reduced following the test-meals. The consumption of the β-glucans enriched pasta for 30 days significantly increased the levels of ghrelin at 0, 15 and 30 minutes after intake. As shown by AUC, on the whole after 30 days treatment we observed an increased release of ghrelin.

PYY (Figure 4). The consumption of test meal induced an increase in plasma PYY concentrations starting from 15 min up to 90 min, peaking at 90 min. The consumption of the β-glucans enriched pasta for 30 days significantly increased the levels of PYY at 0, 15, 30, 60 and 90 minutes after intake. As shown by AUC, on the whole after 30 days treatment we observed an increased release of PYY.

GLP-2 and CCK (Figure 5). Plasma CCK concentrations were lower in fasting state and they were increased following the test-meals whereas no difference were observed for GLP-2 plasma concentrations. However, the consumption of the β-glucans enriched pasta for 30 days did not affect plasma concentrations of GLP-2 and CCK in fasting state or after the meal test.

Figure 5.(A) Plasma concentrations of GLP-2 at day 0 and after 30 days of consumption of enriched β-glucans pasta. (B) Plasma concentrations of CKK at day 0 and after 30 days of consumption of enriched β-glucans pasta. (C) The AUC of GLP-2 during a 3-hours monitoring period. (D) The AUC of CKK during a 3-hours monitoring period. Data are mean values ± S.E.M. (n=10/group).

4.3. Analysis of microbiota in faeces

The analysis of microbiota in stool samples was carried out to identify a possible variation between the baseline and the end of the nutritional intervention. No PCR inhibitors were detected in the reactions. At the end of the intervention, after 30 days, Bifidobacterium Longum, one of the most important probiotic bacterium in human intestine, increases in 80% of subjects analysed (8/10), from 2 to 36-fold than the baseline, while did not show variation in 10% (1/10) and an 8-fold decrease in the other 10% (1/10). Enterococcus faecium, a human pathogen responsible of many nosocomial diseases, surgical wound infection, endocarditis and urinary tract infection, showed a decrease in 90% (9/10), while no result was detected for one sample. However, these results, although interestingly, were not significant.

5. Discussion

The intestinal tract is the largest endocrine organ in the body secreting a wide array of hormones and neuropeptides that regulate the endocrine-metabolic answer to food ingestion within as well as outside the gut. Recent interest in gut hormones has risen with the idea that they modulate glucose tolerance and food intake through a variety of mechanisms, and such hormones are therefore excellent therapeutic candidates for the treatment of type 2 diabetes and obesity [20]. The most widely studied appetite modulating intestinal hormones are GLP-1, GLP-2, CCK, PYY and the peptide ghrelin [21-23]. So far, ghrelin is the only known circulating orexigenic satiety-related hormone and exogenous administration of ghrelin has been shown to stimulate appetite and food intake both in rats and in humans [24]. In contrast to ghrelin, GLP-1, GLP-2 and PYY are secreted into the circulation following meal ingestion and decrease with fasting [25]. The administration of GLP-1 decreases hunger and energy intake both in animals and humans [26]. The post-prandial production of GLP-1 is blunted in severely obese people but controversy exists if GLP-1 concentrations tend to normalize following body weight reduction [27, 28]. Pharmacological activation of the GLP-1 system has beneficial effects on obesity-induced alterations of lipoprotein metabolism. The role of GLP-2-related pathways on lipid levels and metabolism are less clear, but some effects are opposite to GLP-1 [29, 30]. The PYY is another intestinal hormone that is stimulated by food ingestion, especially by the fat content of meal. The infusion of PYY seems to reduce food intake in normal-weight and obese humans; in rodents, repeated infusion of PYY attenuates weight gain [31, 32]. Also CCK is anorexigenic, leading to decrease in food intake [33].

In the present study, we demonstrated that pasta enriched by 6% of β-glucans strongly modified short-term hormones release by intestinal tract. In response to 30 days of pasta intake we observed different postprandial responses of ghrelin, CCK, PYY, and GLP-1. In particular, 30 days

of regular intake of enriched β-glucans pasta significantly increased the endogenous levels of GLP-1 compared with day 0 as well as those of plasma PYY levels. Furthermore, plasma ghrelin concentrations were higher in fasting state and they were reduced following the test-meals. So, food stimulated postprandial secretion of the anorexigenic hormones PYY, and GLP-1 and repression of concentrations the orexigenic hormone ghrelin that have been proposed to increase satiety and decrease short-term food intake [34]. As stated in the Introduction, it has been claimed an effect on satiety by products prepared from barley flour enriched with β-glucans, but in that study no hormonal measurement was performed. Thus, to the best of our knowledge, the present study is the first one to demonstrate an effect of intestinal hormones.

Due to the known effects of these hormones in weight regulation, we can suggest that the regular intake of pasta added with β-glucans should be a promising tool to prevent obesity.

On the other hand, it is well known that harmful metabolites, produced by the human microbiota, likely participate to ageing process, contributing to inflammageing and immunosenescence. Although the intestinal microbiota is relatively stable throughout adulthood, age-related modifications in the intestinal tract, as well as changes in diet and host immunity reactivity, inevitably affect bacteria population composition. The prolonged retention time leads to an increase in bacterial protein fermentation and consequently putrefactive processes in the gut with a greater susceptibility to inflammatory diseases [35]. Hence, the gut ecosystem shows the potential to become a promising target for strategies able to contribute to the health status of elderly. In this context, the consumption of prebiotics as β-glucans fibres might be useful in both prevention and treatment of age-related pathophysiological conditions, favouring the attainment of longevity [20]. Accordingly, we observed changes in microbiota composition in our subjects, after 30 days treatment with pasta added with β-glucans, however, the brief period of time of our study did not allow us to reach significant changes.

It is to note that our study presents some limitations. One is strictly related to the intrinsic complexity of human as study model and to the inter/intra-individual variability. More important, we can consider this study only a pilot one due to the small size sample (10 people)), which is not enough to completely cover the genetic and individual variability. Also the lack of a real control group can be considered a weakness of our study but the possibility to administrate a placebo (pasta without β-glucans) couldn't be an option because of the totally different organoleptic properties (in particular taste and colour). Thus, here we presented preliminary results but further analyses are needed to confirm our data.

However, present and previous results clearly suggest that the regular intake of this food might useful for the prevention of obesity, type 2 diabetes and metabolic syndrome [6]. So, The use of

pasta added with 6% β-glucans seems exhibit promising responses on glucose metabolism, LDL-cholesterol values, intestinal hormones responses and microbiota modification.

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Author Contributions: Design and conduction of the study :Sara Baldassano, Silvio Buscemi, Sonya Vasto; data collection and data interpretation: manuscript drafting: Sara Baldassano, Sonya Vasto. Calogero Caruso made critical revisions to the draft . All authors read and approved the final manuscript..

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	day 0	day 30	P[1]
Gender (Females/Males)	5/5	5/5	
Age (years)	26.5±2.5		
Body weight (kg)	84.8±8.1	84.2±6.5	
BMI (kg/m2)	27.4±3.4	27.3±3.3	
Fasting blood measurements:			
Glucose (mg/dl)	99 ± 2	89 ± 9	= 0.05
Total cholesterol (mg/dl)	175 ± 24,7	160 ± 13,3	= 0.03
HDL-cholesterol (mg/dl)	52 ± 7,5	52 ± 5,1	= 0.8
LDL-cholesterol (mg/dl)	103 ± 20,7	91 ± 10,4	= 0.03
Triglycerides (mg/dl)	96 ± 32,7	82 ± 26,3	= 0.3

Table 1. Characteristics of the cohort and blood measurements at day 0 and 30 days after regular consumption of pasta added with β-glucans

All data are presented as means ± S.D.

[1] Wilcoxon test.

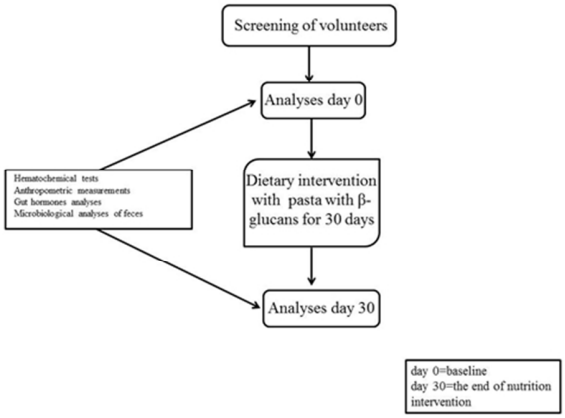

Figure 1. The flow chart of study design.

216x121mm (96 x 96 DPI)

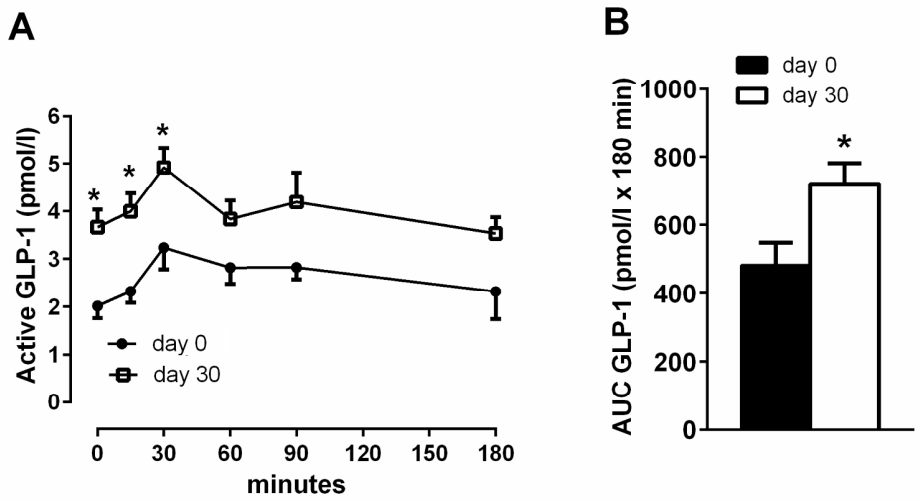

Figure 2. (A) Plasma concentrations of active GLP-1 at day 0 and after 30 days of consumption of enriched β-glucans pasta. (B) The AUC of active GLP-1 during a 3-hours monitoring period. Values are mean ± S.E.M. *p <0.05. (n=10/group).

569x364mm (96 x 96 DPI)

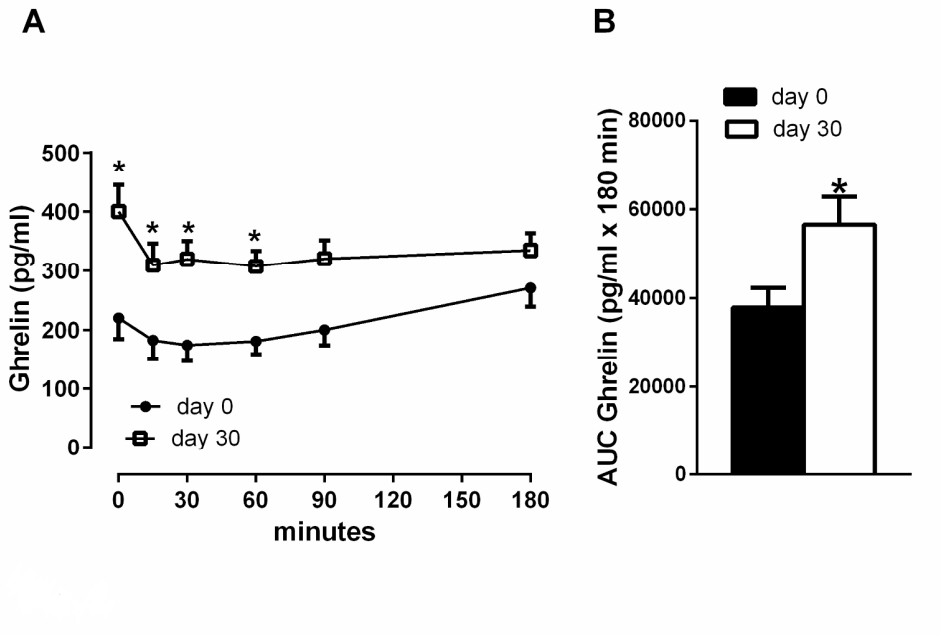

Figure 3.(A) Plasma concentrations of ghrelin at day 0 and after 30 days of consumption of enriched β-glucans pasta. (B). The AUC of ghrelin during a 3-hours monitoring period. Data are mean values ± S.E.M. *p<0.05. (n=10/group).

209x140mm (300 x 300 DPI)

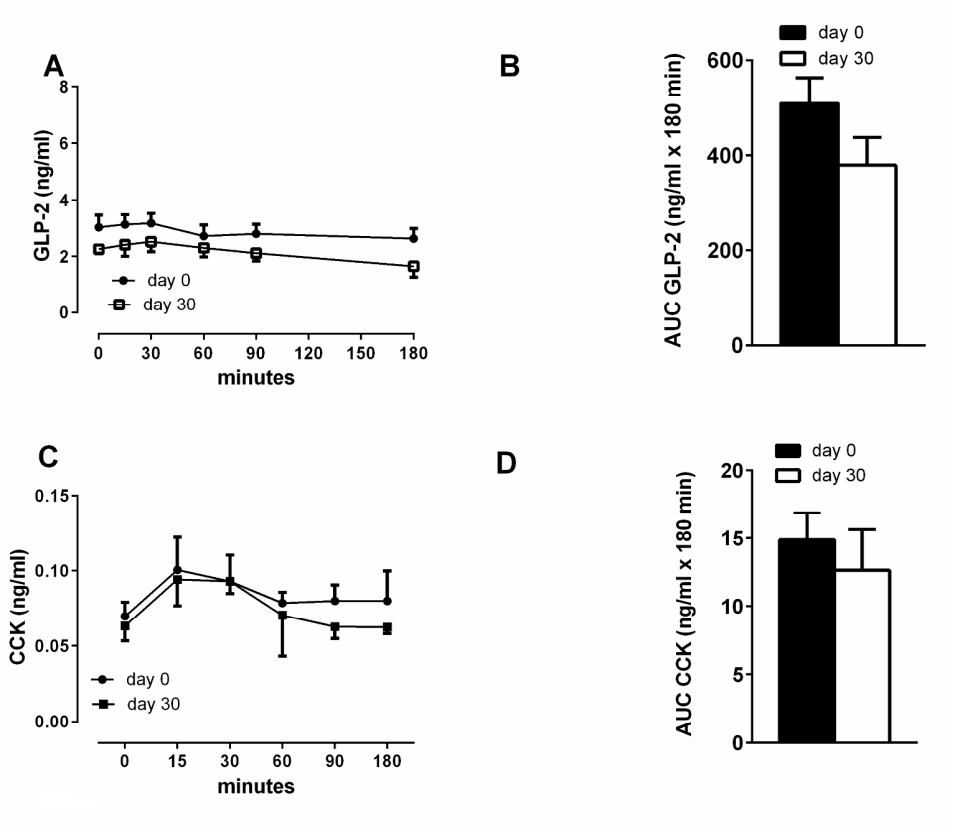

Figure 5.(A) Plasma concentrations of GLP-2 at day 0 and after 30 days of consumption of enriched β-glucans pasta. (B) Plasma concentrations of CKK at day 0 and after 30 days of consumption of enriched β-glucans pasta. (C) The AUC of GLP-2 during a 3-hours monitoring period. (D) The AUC of CKK during a 3-hours monitoring period. Data are mean values ± S.E.M. (n=10/group).

248x213mm (300 x 300 DPI)

VIII. Pasta with *Opuntia Ficus Indica*: a functional food with anti-inflammatory, hypoglycemic and antioxidant effects

VIII.I Introduction

The increased interest in the health benefits of food has stimulated research studies to look beyond the basic nutritional values of foodstuffs towards disease prevention. In this regard, the beneficial effects of plant remedies are generally accepted and dependant on the presence of bioactive compounds from the whole plant, part of plant or processed foods obtained with plant extracts.

OFI, belonging to the family *Cactaceae*, is commonly known for its nutritionally rich edible fruits, the prickly pears, often investigated for their antioxidant and anti-inflammatory properties. However, its leaves, the cladodes, are also used as fresh green vegetable and in salads.

This cactus plant is well adapted to arid lands and to diverse climates, including Mediterranean basin, Middle East, South Africa and America, Australia and India, and in some countries is utilized in the food and cosmetic industries (*Saenz, 2000*).

OFI, fruits and stems, have traditionally been used for several medicinal purposes (*Hunt et al., 2006*) and, nowadays, a lot of pharmacological interventions have suggested its health benefits for the prevention of some chronic diseases (*Osuna et al., 2014*). Recently, the interest in the role of OFI in the

control of obesity and other metabolic parameters, such as glycaemia and lipid profile has increased. However, although some of these results have been proven in animal tests (rabbits, mice) and mainly concern the properties of prickly pears, they are disputable in human studies. Indeed, a recent meta-analysis showed that few clinical trials evaluating the effects of OFI on human beings have been published. The supplementation with OFI generates no statistically significant effects on body weight in these trials but its consumption would seem to be associated with a reduction of body fat, blood pressure and TC (*Onakpoya et al. 2014*). Nevertheless, some studies appear inconsistent and further clinical interventions are necessary to evaluate the real contribution of OFI to human health.

The aim of our study was to evaluate the effect of a pasta, made by University of Palermo, with OFI cladode extracts on hematochemical, anti-inflammatory, antioxidant, anthropometric and microbiological parameters, to test its potential hypoglycemic, hypocholesterolemic and weight loss properties and its possible modulator action on gut microbiota (prebiotic like effect).The idea was to manufacture a processed food commonly used by Mediterranean people with added cladode extracts that represent a source of phytochemical

compounds, such as phenolic acids (malic acid) and flavonoids (*Ginestra et al., 2009*). The cladodes are mainly comprised of water (80-95%), carbohydrates (3-7%), fibers (1-2%), and protein (0,5-1%). The carbohydrate component includes polysaccharides like mucilaginous, whose role is to regulate the plant cellular water, and pectins (*Zhao et al., 2007*). Their utilization in food, medical and industrial applications was also documented. In Mexico, the cactus pads are commonly served with meals, similar to green beans (*Feugang et al., 2006*; *Stintzing et al., 2005*). Moreover, the reported heat-resistance of cactus pear extract antioxidants, suggests the capability of these antioxidants to maintain their activity well after harvest and during adequate storage (*Siriwardhana and Jeon, 2004*).

The statistical data analysis, shown in part in this thesis, is ongoing and the results displayed are preliminary.

VIII.II Materials and methods

Study design

The trial consisted in the consumption of 500 g/week of pasta with 3% of OFI cladode extract, for 30 days. The pasta was made by University of Palermo, using *Vertola* wheat with a small amount of *Pietra Fitta* wheat. OFI extract was used in

the gel form, replacing all of the water used for the pasta dough. Extract and pasta characterization was conducted respectively by Professor Bonina (University of Catania) and Professor Cirrincione (University of Palermo). The extract was composed of 30% insoluble polysaccharides and had a strong antioxidant power (data unpublished). See **Figure 10** for the flow chart of the study design.

Subjects recruited

We have performed a nutritional intervention on 42 randomized volunteers (mean age 47,05), both men and women, recruited from September 2015 to December 2015. The subjects included were: aged between 19 and 69, Caucasian, and with metabolic syndrome. Inclusion criteria were: slight dyslipidemia (TC190-240 mg/dL, triglyceride level ≥150 mg/dL); waist circumference ≥102 cm in men and ≥88 cm in women; altered glucose tolerance (fasting blood glucose ≥100 mg/dl); any treatment for specific disease, including drugs to treat metabolic disorders. Exclusion criteria were: a diagnosis of a severe systemic diseases; a history of use of any pre or probiotics as dietary supplements within 3 months prior to the study; a history of treatment with statins or similar,

and with lyposoluble drugs; the onset of gastrointestinal disorders or the use of antibiotics during the nutritional intervention; restrictive dietary requirements. No restriction related to sex was considered. Enrolled subjects underwent a complete nutritional anamnesis with trained nutritionists. Two subjects dropped out of the trial. One was excluded. All participants signed an informed consent before the enrolment. To respect the privacy, everyone was identified with an alphanumeric code. Height and weight were measured wearing light clothes and barefoot. The body composition was registered using specific hardware and software. Body mass index was calculated as weight (in kilos) over height squared (in square metre). Dietary habits were assessed through a food frequency questionnaire, officially validated by the EPIC study. Blood tests, complete lipid profile, oxidative stress, cytokines analyses were carried out for all subjects at T0 and T1. Molecular analyses were conducted on total DNA obtained from faecal samples to measure the variation of its amount. A database was created to insert all participants' data and to handle the collected information.

Hematochemical tests

See *Accardi et al., 2016b*.

Pro and anti-inflammatory cytokines analyses

Cytokines analysis of high sensitivity IL-6, IL-10, IL-1B, IL-17A, and high sensitivity tumor necrosis factor (TNF)-α were measured. See *Accardi et al., 2016* b for the methods.

Evaluation of parameters of oxidative stress

These analyses were conducted in collaboration with the University of Sassari. Oxidative stress markers (thiobarbituric acid reactive substances, TBARS, and plasma proteins SH, PSH) and antioxidants (paraoxonase, PON, and glutathione, GSH, activity) were measured in 39subjects with metabolic syndrome. TBARS were determined according to the method described by Esterbauer and Cheeseman (*Esterbauer and Cheeseman, 1900*).TBARS methodology measures MDA and other aldehydes produced by lipid peroxidation induced by hydroxyl free radicals. Plasma was mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid and heated at 95°C in a thermoblock heater for 25 min. TBARS were determined by measuring the absorbance at 535 nm. A

calibration curve was obtained using standard MDA and each curve point was subjected to the same treatment as that of the samples. PON activity was determined by measuring the increase in absorbance at 412 nm (formation of 4-nitrophenol) using paraoxon (O,O diethyl-O-p-nitrophenyl phosphate) as a substrate (*Gan et al., 1991*). Enzyme activity was calculated by using the molar extinction coefficient of 17.100 $M^{-}1cm^{-}1$ and one unit (U) of PON activity was defined as 1 nmoL of 4-nitrophenolformed per minute. Plasma PSH determination was performed by spectrophotometry with 5.5'-dithiobis-2-nitrobenzoic acid (DTNB) as titrating agent by measuring the absorbance of conjugate at 405 nm (*Ellman, 1959*). Concentration in samples was determined from a GSH standard curve.

Microbiological and molecular analyses on feces

Total DNA was purified from faecal samples using the QIAamp DNA Stool Minikit (Qiagen, Germany) following manufacturer's instructions. The purified DNA was spectrophotometrically quantified at 260nm by means of NanoDrop 2000c (Thermo Scientific) and stored at -80°C until use.

The relative abundance of *Lactobacilli* and *Enterobacteriaceae* 16S rRNA gene was measured by quantitative PCR (qPCR), using the primer pairs described in **Table 3**. A known amount of exogenous *Anemoniaviridis* (*Cnidaria*) DNA was added to template faecal DNA and the single copy gene AvHSP28.6 was amplified as internal reference sequence to overcome the lack of unbiased target among samples (*Nicosia et al., 2014*; *Gagliano et al., 2016*). The 25 µl of qPCR mixtures contained 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µM each of the forward and reverse primers, 20 ng of faecal DNA and 5 ng of exogenous DNA. The reactions were carried out on a ABIPRISM 7500 System (Applied Biosystems, Forster City, USA) with initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 60 s, followed by a melting curve from 60 to 95 °C. All qPCRs were run in triplicate. Melting curve and gel electrophoresis analyses were performed to confirm the specificity and the appropriate size of the amplified products. Data analysis was performed according to the $2^{-\Delta\Delta CT}$ method (*Schmittgen and Livak, 2008*). All data represented relative mRNA expressed as the mean ± standard deviation (n=3). Significant differences between values of

different treated groups and the reference control groups were determined by one-way ANOVA with Tukey's post-test.

Enterobacteria	5′- ATGGCTGTCGTCAGCTCGT-3′ F 5′- CCTACTTCTTTTGCAACCCACTC-3′ R
Lactobacilli	5′-GCAGCAGTAGGGAATCTTCCA-3′ F 5′-GCATTYCACCGCTACACATG-3′ R

Table 3. Oligonucleotide primers used in this study.

Statistical analyses

The multiple logistic regression analyses are ongoing. However, the preliminary comparisons were performed with the Student's t-test or the Wilcoxon signed rank test, according to the normality of samples.

VIII.III Preliminary results and discussions

Hematochemical tests

Physical characteristics and statistically significant biochemical parameters are shown in **Table 4**. In particular, at T1, in analyzed subjects, uric acid significantly decreased,

together with a decrease of nitrogen, probably due to an increase of fiber intake during the nutritional intervention that facilitates the elimination of the waste from the intestine. Uric acid, the last product of purine metabolism, is a biomarker of inflammation. Its reduction suggests a supposed anti-inflammatory properties of this pasta. We also noted a significant decrease of glycaemia that suggests a hypothetic hypoglycemic effect. All these values, increased or decreased, were in normal range. All the other biochemical values did not statistically change following the 30-days consumption of pasta added with OFI, although a trend towards a decreased TC was observed (212 at T0 vs 206,07 at T1), according to literature data.

Anthropometric measurements

At the end of the intervention, body weight and body mass index (BMI) did not vary as well as fat and muscle mass along the 30 days. However, in the analysed subjects, the abdominal and the thoracic waist decreased, suggesting a reduction of localized adipose tissue probably due to the over intake of fibers. After all, few study showed the anti-obesity effect of OFI extracts and, to the best of our knowledge, no

studies were conducted regarding the role of processed food realized with these extracts.

Cytokine analysis

The serological analysis of the levels of the main pro and anti-inflammatory cytokines was conducted. At the end of the intervention, no significant changes were observed, suggesting that pasta with *Opuntia* does not have any effect on cytokines status probably due to the short duration of the intervention.

Oxidative stress analysis

The antioxidant effect of OFI was demonstrated but only a few studies showed this property on humans. Most of them have utilized OFI supplement and no functional food. Thus, we have analysed the effects of pasta added with *Opuntia* extracts intake on some oxidative stress parameters. In particular, we noted that the PON plasma levels and GSH in red blood cells significantly increased, confirming the anti-oxidant power of OFI cladode extracts also in processed foods. This is probably due to high polyphenol presence. However,

MDA levels, the main product of the PUFA peroxidation and an important index of oxidative stress, was unchanged.

Microbiological analysis on feces

The development of real-time quantitative PCR procedures provides useful tools for the accurate assessment of specific components of microbial communities and their ratios. In order to overcome any random variation in qPCR efficiency and sensitivity due the occurrence of inhibitors, such as bilirubin and bile salts, which could impair the analyses outcome, serial dilutions of pooled faecal DNAs from both T0 and T1 subject were prepared and mixed with fixed amount of exogenous *Anemoniaviridis* DNA. The amplification efficiency of AvHSP28.6 was evaluated and a template amount ensuring reproducibility was identified (data not shown). As a result, in our experiments 16S DNA amplifications was obtained for all the subjects except for 4 probands. The amount of *Lactobacilli* in faecal samples was evaluated and a significant reduction was noticed before the intervention in 30 probands, while an increased amount, ranging from 2.3- to 95-fold higher than corresponding controls (T0), was detected in five subjects.

Moreover, in order to evaluate the specificity of the observed pattern, similar assays were carried out to evaluate the amount of *Enterobacteriaceae* in the same specimens. As previously observed, the qPCR analyses revealed a decrease of *Enterobacteriaceae* among probands at T1. Thus, it could be hypothesised that an overall reduction in the amount/composition of the intestinal microbiota maybe associated to dietary supplementation of compounds from OFI (data not shown).

Extended dietary interventions and larger cohort will be useful to confirm the *Lactobacilli* increase in responding probands.

Variable	T0	T1	p-value	%
Uric Acid	4.98	4.73	0.014	-5%
Nitrogen	32.15	30.00	0.03	-6.70%
Glycaemia	84.02	80.89	0.031	-4%
TC	212.05	206.07	/	-2.82%
Abdominal waist	92.23	90.66	0.0009	-2%
Thoracic waist	102.92	101.51	0.00017	-1%
PON	915.06	997.06	0.012	+8.96%
GSH	1020.5	1147.1	0.019	+12.40%

Table 4. The table shows the arithmetic average values at T0 and T1, the p-value and the variation in percentage (+ and – indicate, respectively, an increase and a decrease of the variables at T1).

VIII.IV Discussion and conclusions

It is well known that the MedDiet and Mediterranean functional foods can positively influence the health of each individual. In particular, it is know that fibers have an important role. In the present study, we preliminary demonstrated that the daily intake of pasta made with 3% of OFI extracts, rich in fibers and bioactive compounds, has a strongly antioxidant effect. Moreover, this pilot study has shown the hypoglycemic property of this processed food as well as its effect on the abdominal and thoracic waist. It also was observed an anti-inflammatory effect linked to the uric

acid reduction and a positive decrease trend of TC. Further analyses are necessary to confirm the *Lactobacilli* increase and the reduction of *Enterobacteriaceae* in responding subjects, probably associated to dietary supplementation of OFI.

It must be stressed that the study presents limitations, concerning: the average age of recruited people, the small size of population analysed and the short duration of the intervention. This is a good choice in terms of compliance to the study because the longer the duration of the intervention the greater the drop out effect. But, a long-term dietary intervention could be stronger in terms of variation of analysed parameters (e.g., prebiotic effect).

According to the results of this pilot trial, we might speculate that a system of well-balanced diet of carbohydrates and fibers like OFI, as the Mediterranean diet would appear to be, may be suitable for helping accumulation due to age and foods or for maintaining normal glycaemic values.

For all its proven properties, pasta with OFI extracts can be considered a functional food and it will present an important opportunity for the food and farming industry, especially in Sicily, where the OFI represent a widespread, economic resource.

IX. Antioxidant effects of extra virgin olive oil polyphenol extracts on peripheral blood mononuclear cells

IX.I Introduction

As we have extensively explained in this thesis, several epidemiological studies have shown a relationship between the MedDiet and the reduction of the onset of age-related pathologies such as CVDs, cancers, and diabetes. Several of these reports attribute these health benefits to high consumption of bioactive molecules, greatly present in this dietary pattern that has, as common element between all Mediterranean countries, EVOO. It is rich in phenols and flavonoids, bioactive compounds also related to healthy ageing (*Rigacci and Stefani, 2016*; *Tripoli et al., 2005*).

Among these, the most studied and potentially promising are oleuropein and its hydrolysis products, such as 3, 4-dihydroxy-phenylethanol (hydroxytyrosol) that have established anti-oxidant properties. These compounds are present in olive leaves, drupes and virgin olive oils, and are responsible for olive oil's characteristic bitter taste and its stability (*Andrewes et al., 2003*). Oleuropein is the most studied phenolic compound found in EVOO. It shows anti-oxidant activity and antimicrobial effect especially on a broad range of Gram-positive bacteria (*Fleming et al., 1973*; *Kruk et al., 2005*). Hydroxytyrosol is the main polyphenol present in free form in

EVOO. It acts as a scavenger of ROS and exerts beneficial cardiovascular effects through the decrease of oxidized LDL and platelet aggregation. It would seem that hydroxytyrosol stimulates Sirt1 activation and increases antioxidant defences via nuclear factor-E2-related factor 2 (Nrf-2) induction in mice fed with a diet rich in olive oil phenolics (*Bayram et al., 2012*). Furthermore, oleuropein mediates Sirt activation but it acts by reducing adipogenesis and inducing autophagy via AMP-activated protein kinas (AMKT) activation or, directly, through the modulation of insulin/IGF1/protein kinase B and mTOR pathways. This determines forkhead box O (FOXO) 3A stimulation with ensuing transcription of homeostatic genes that favor longevity and reduce inflammatory states (*Rigacci et al., 2015*; *Svobod et al., 2014*). In this way, these polyphenols act as CR mimickers.

Moreover, oleuropein and its derivatives can act as anti-inflammatory molecules, positively contrasting the chronic low-grade inflammation and the accumulation of ROS, typical of age-related diseases (*González-Correa et al., 2009*). In a study, oleuropein has been shown to enhance NO production in macrophage cells, thus to fight against endotoxins and facilitate the elimination of pathogens (*Durlu-özkaya and Özkaya,*

2011). Several studies have demonstrated that these compounds are able to inhibit proliferation and induce apoptosis in different tumor cell lines (*Fabiani, 2016*) .So, for all these reasons, they arouse a great interest in the scientific community.

Although several studies have shown the effects of EVOO polyphenols on cell lines or animal models, few interventions have been conducted in humans both *in vivo* and *ex vivo* studies. Since the beneficial effect of EVOO is associated to the simultaneous action of all compounds that it contains, to study the single effect of the oleuropein or its derivatives, their extracts and derivatives are used. In particular, it has been reported that some acetyl derivatives of phenols maintain biological antioxidant activity, compared to that of the unprotected compound, probably because of the massive deacetylation by carboxylesterases. Moreover, it was demonstrated that this chemical modification improves the capacity of the molecule to permeate the cellular membrane (*Procopio et al., 2008*).

The aim of this study will to test the antioxidant and anti-inflammatory activities of oleuropein, hydroxytyrosol and

their paracetylated derivates on PBMCs, using an *ex vivo* cellular screening system.

IX.II Materials and methods

Subjects recruited

We recruited fourteen healthy randomized Caucasian volunteers (aged 23-65), from April to July 2016. No restriction related to sex was considered. All participants signed an informed consent before the enrolment. To respect their privacy, everyone was identified with an alphanumeric code. A database was created to insert all participants' data and to handle the collected information.

Isolation of PBMCs

Donor PBMCs were isolated from leukocyte-enriched human peripheral blood collected in specific blood collection tubes containing ethylene diamine tetra acetic acid (EDTA). The cells were separated using Ficoll density gradient centrifugation, washed twice in PBS, and stored in 10% heat-inactivated fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) at -80 °C for further tests. For the analyses, the viable cells (95–98% of the preparation) were re-suspended at the

concentration of 1×10^6/ml in complete medium composed of Roswell Park Memorial Institute (RPMI) 1640 (Euroclone, Devon, UK) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 25mM 4-2-hydroxyethyl-1-piperazine ethane sulfonic acid (HEPES), and 1 mM L-glutamine.

Tested compounds

On PBMCs we already aglicone oleuropein paracetylated (AOP). We tested, partly, oleuropein, hydroxytyrosol, and hydrossytyrosol paracetylated (HP). These compounds were obtained as described *Procopio et al., 2009*. Paracetylated derivatives were achieved after chemical modification of oleuropein following a patented synthetic method (*Procopio et al., 2008*). Before cell cultures tests, the lyophilized extracts were suspended in ethanol.

PBMCs viability assay in ethanol and in polyphenols

The cells were subjected to a viability assay in ethanol to identify the non-toxic ethanol concentration for the PMBCs to use to suspend lyophilized polyphenol extracts. The PBMCs were cultured in 96-well flat-bottom plates at 150.000 cells per well in complete RPMI with different ethanol concentrations

(5, 2.5, 1, and 0.5%). We evaluated cell viability using Trypan blue dye exclusion assay. After 24, 48, and 144 hour treatments, the PBMCs was counted in the Burker chamber. Cell growth rate was calculated as percent of non-stained (live) cells over control. Cell mortality was calculated as the percentage of stained cells over total cells and expressed as a ratio between treated and untreated (control).

Then, cells were cultured and incubated with the polyphenol extracts. We used three different polyphenol concentrations (1, 10 and 100 μM), and we treated the cells for 24 and 48 hours. Subsequently, we added, to each well, 250 μM hydrogen peroxide (H_2O_2), to induce oxidative stress, for 30 minute and, immediately after, we evaluated the ROS production by Synergy-HT plate-reader (Ex/Em 488/528nm), after a treatment with 20mM of 2',7'-dichlorofluorescin diacetate.

IX.III Preliminary results and discussion

At the time of writing of this thesis, the experiments were ongoing and only some anti-oxidant tests were conducted.

For the re-suspension of lyophilized polyphenols extracts, we chose 0.5% ethanol concentration because after 144 hours the cell viability was greater than 100%, suggesting

that this ethanol amount probably stimulates cell growth (**Figure 11**).

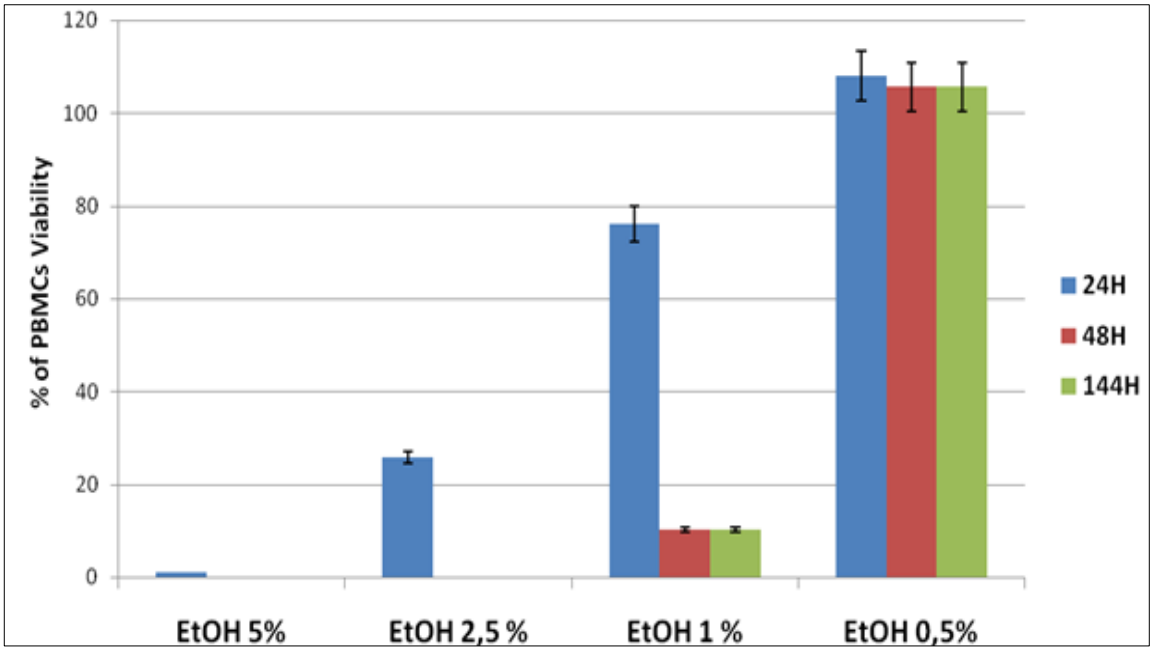

Figure 11. The histograms show the PBMCs viability after the EtOH assays.

About anti-oxidant tests with polyphenol extracts, preliminary results demonstrate that the AOP works in a dose-dependent manner, scavenging the ROS after 24 hours of treatment (**Figure 12**). The same is not true after 48 hours, when the most efficient concentration is the 10 μM (**Figure 13A**).

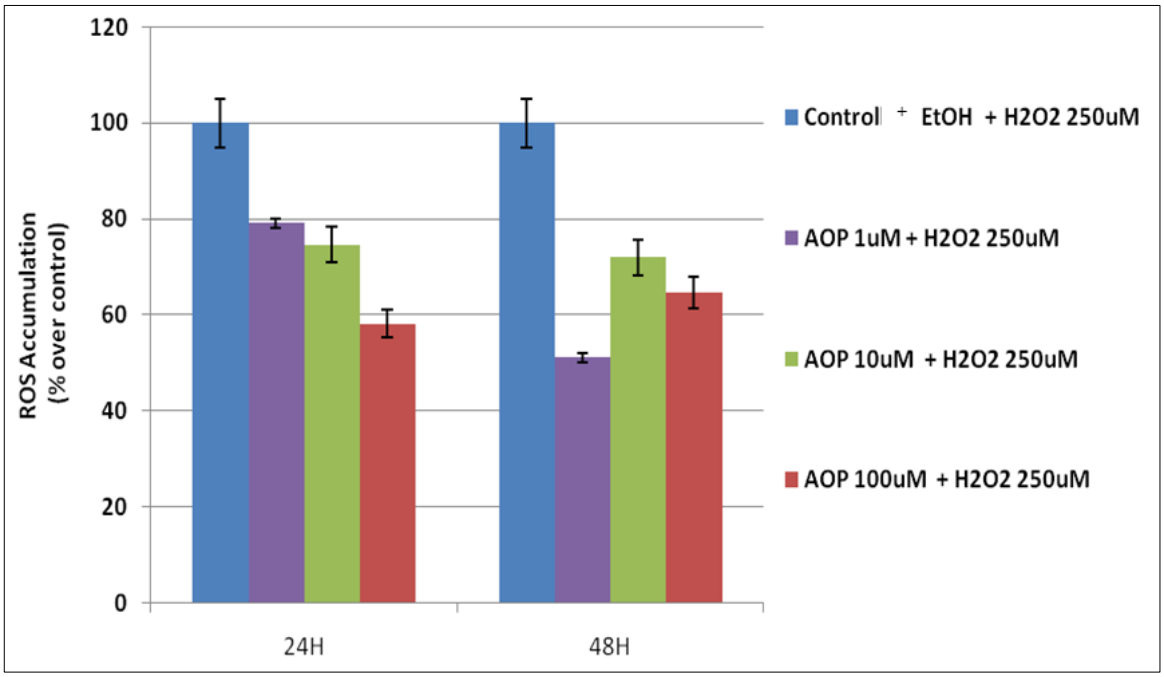

Figure 12. The figure shows the percentage over the control of the ROS accumulation in one sample treated with H_2O_2 and AOP at 24 and 48 hours. As the bars show, the effect of AOP is dose-dependent, at 24 but not at 48 hours.

An interesting observation is that after one hour of reading of the fluorescence in the 24 hour treated sample, the 100 μM concentration works less than the 1 μM an 10 μM (**Figure 13B**) but the effect became dose-dependent after 2 hours reading (**Figure 12**) (data unpublished).

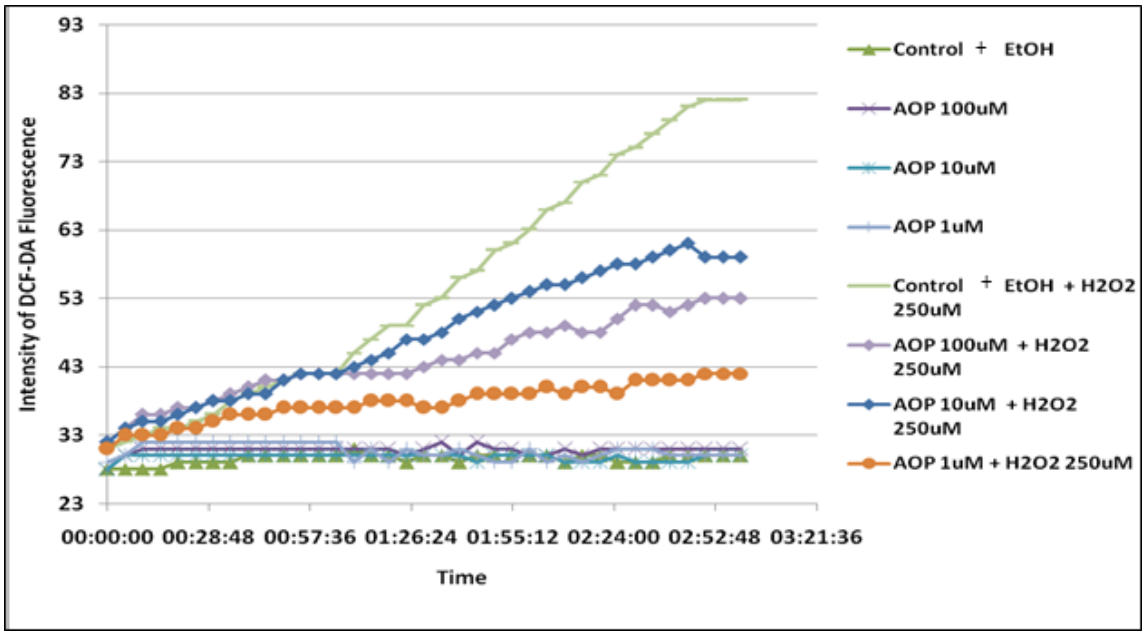

Figure 13A. The figure shows the result obtained in one sample of PBMCs treated with H_2O_2 and AOP at different concentrations for 48 hours, after 1, 2 and 3 hours of fluorescence emission.

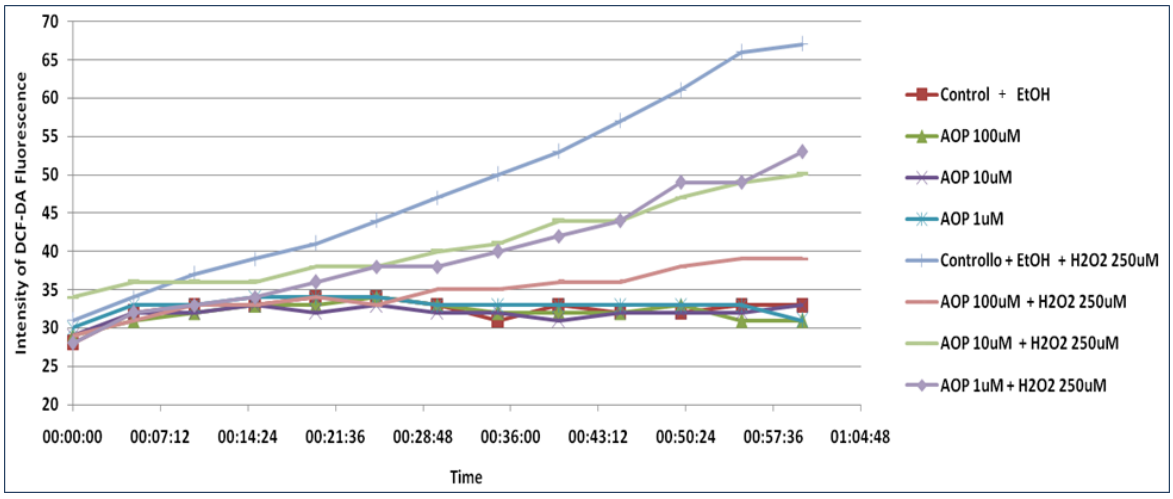

Figure 13B. This figure shows the intensity of dichlorofluorescin diacetate fluorescence dependent on the time. The figure also shows the result obtained in one sample of PMBC treated with H_2O_2 and AOP at different concentrations for 24 hours, after 1 hour of fluorescence emission.

IX.IV Conclusions

Although it is necessary to verify these data in a larger sample of individuals, these preliminary results are very interesting.

Some studies have demonstrated the antioxidant activity of olive oil phenols in several *in vitro* systems but few and conflicting studies analysed their role in *ex vivo* model (*Fabiani, 2016*). In a study was investigated the potential protective activity of different olive oil phenols on oxidative DNA damage induced by H_2O_2. The authors showed a potent DNA damage preventive activity of olive oil phenols, providing new evidence to support a possible role of these compounds in the prevention of cancer (*Fabiani et al., 2008*). Another investigation demonstrated that the hydroxytyrosol has a promising antioxidant potential in protecting the PBMCs against 2,3,7,8-tetrachlorodibenzo-p-dioxin, a substance that induced oxidative stress (*Ilavarasi et al, 2011*).

It was previously reported that the acetyl derivatives of phenolic compounds maintain biological antioxidant activity compared to that of the parent compound, probably because of extensive deacetylation of hydroxytyrosyl acetate by carboxylesterases (Mateos et al., 2005). Such deacetylation can

take place either within the cell, upon absorption of the acetylated molecule, or in the extracellular space by secreted esterases, generating free hydroxytyrosol, which is the effective antioxidant compound. *Procopio et al*. demonstrated that peracetylation of oleuropein and its derivatives may improve their capacity to permeate the molecular membrane and their "drug likeness" (*Procopio et al., 2009*). The authors investigated the antiproliferative and antioxidant effects of peracetylated oleuropein, peracetylated aglycone and peracetylated hydroxytyrosol, compared to unprotected oleuropein, on two lines of human breast cancer cells, showing antiproliferative and antioxidant effects.

According to *Procopio*, in our pilot study we showed that paracetylated molecules are chemically more stable derivatives that are able to be biochemically converted *in vivo* into their original active forms. However, this hypothesis this requires further analyses.

Nowadays, the use of oil-based supplements is widely popular but the real effects on humans are not always tested. Their application in terms of strengthening of therapies or prevention is interesting but, because they are chemical

molecules, it is important to characterize their possible positive and adverse effects using human models, firstly *ex vivo* ones.

X. Nutrigerontology: a key for achieving successful ageing and longevity

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Nutrigerontology: a key for achieving successful ageing and longevity

Anna Aiello[1], Giulia Accardi[1], Giuseppina Candore[1], Giuseppe Carruba[2], Sergio Davinelli[3], Giuseppe Passarino[4], Giovanni Scapagnini[3], Sonya Vasto[5] and Calogero Caruso[1*]

Abstract

During the last two centuries the average lifespan has increased at a rate of approximately 3 months/year in both sexes, hence oldest old people are becoming the population with the fastest growth in Western World. Although the average life expectancy is increasing dramatically, the healthy lifespan is not going at the same pace. This underscores the importance of studies on the prevention of age-related diseases, in order to satisfactorily decrease the medical, economic and social problems associated to advancing age, related to an increased number of individuals not autonomous and affected by invalidating pathologies. In particular, data from experimental studies in model organisms have consistently shown that nutrient signalling pathways are involved in longevity, affecting the prevalence of age-related loss of function, including age-related diseases. Accordingly, nutrigerontology is defined as the scientific discipline that studies the impact of nutrients, foods, macronutrient ratios, and diets on lifespan, ageing process, and age-related diseases. To discuss the potential relevance of this new science in the attainment of successful ageing and longevity, three original studies performed in Sicily with local foods and two reviews have been assembled in this series. Data clearly demonstrate the positive effects of nutraceuticals, functional foods and Mediterranean Diet on several biological parameters. In fact, they could represent a prevention for many age-related diseases, and, although not a solution for this social plague, at least a remedy to alleviate it. Thus, the possibility to create a dietary pattern, based on the combined strategy of the use of both nutraceuticals and functional foods should permit to create a new therapeutic strategy, based not only on a specific bioactive molecule or on a specific food but on a integrated approach that, starting from the local dietary habits, can be led to a "nutrafunctional diet" applicable worldwide.

Keywords: Ageing, Longevity, Mediterranean Diet, Nutraceuticals, Nutrigerontology, Phytochemicals

Background

Nutrigerontology is defined as the scientific discipline that studies the impact of nutrients, foods, macronutrient ratios, and diets on lifespan, ageing process, and age-related diseases. Its goal is to investigate about compounds, foods, and diets that can reduce the risk of ageing-related diseases and increase the healthy lifespan, so achieving successful ageing and longevity [1].

Many definitions of longevity and successful ageing have been proposed but none has been accepted yet. In May 2012, a group of scientists and clinicians met in Athens (Greece) to consider the relevance of ageing, longevity, exceptional longevity and related genetic and non genetic markers. The workshop led to the creation of a consensus statement to highlight the importance of a common view related to these processes, since they represent phenotypes that are rapidly spreading worldwide [2].

As reported in this panel: "Successful ageing involves avoidance (or late onset) of age-related diseases including cardiovascular disease which is the main cause of death, and other organ specific diseases, disability, preservation of desirable cognitive and physical function and social activities throughout the lifespan". Moreover, it proposes the definition of exceptional longevity in relative and absolute terms, on the basis of demographic data. It quotes that: ""Relative" suggests that longevity is concept country/population specific and must take into

* Correspondence: calogero.caruso@unipa.it
[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Palermo, Italy
Full list of author information is available at the end of the article

consideration the life expectancy of the different populations/countries, which show great variability owing to historical, anthropological and socio-economic differences. In "absolute" terms longevity could be defined according to the maximum lifespan attained and scientifically validated by human beings in the planet" [2].

Also, the statement reported the main genes related to longevity and ageing. Such genes and their encoded proteins are included in a variety of signalling pathways, i.e., the nutrient sensing insulin/insulin-like growth factor-1 (IGF-1) or mammalian target of rapamicin (mTOR), the oxidative stress and anti-oxidant ones and that involved in the control of immune-inflammatory responses [2].

In addition, it is becoming clear that epigenetic changes linked to environmental/life style factors (such as physical activity, diet and emotional stress) play a role in longevity attainment [2, 3].

In particular, a nutrient-sensing pathway is activated by nutrients that trigger signals that lead to a downstream activation of genes involved in ageing processes. The complex relationship among nutrition and healthy ageing is not fully understood, but evidences have already demonstrated that both in animal models and humans, dietary intervention can positively modulate ageing process, preventing or decreasing various age-related diseases and their generalized pro-inflammatory status, the inflammageing [4, 5]. So, healthy diets that not overstimulate mTOR and insulin/IGF-1 pathways because poor in refined sugars and animal proteins can substantially reduce the risk of age-related diseases, hence favouring successful ageing and longevity [6, 7]. On the contrary, a bad dietary lifestyle accelerates the ageing process, activating the pathways with nutrients, growth factors and mitogenic stimuli, so accelerating ageing phenotype [1].

Mediterranean Diet (MedDiet) is one of the most studied healthy dietary patterns. It is an alimentary regimen with low-glycaemic index and low animal protein intake that contains phytochemical compounds found in vegetables, fruits, red wine, olive oil or nuts, with anti-inflammatory and anti-oxidant effects. This pattern, that more than a diet could be defined a lifestyle, owes its health properties also to the nutraceuticals [1, 8–11], defined as "naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products, and have health promoting, disease preventing, or medicinal properties". The term, coming from the conjunction among nutrition and pharmaceutics, was coined in 1989 by Stephen De Felice [12].

Dietary phytochemicals belong to this group. They are non-nutrients compounds from a wide range of plant-derived foods. Despite the translational gap among basic and clinical research, the current understanding of the molecular interactions between phytochemicals and immune-inflammatory and oxidative stress pathways could help in designing effective nutritional strategies to delay ageing and age-related diseases. In particular, it has been claimed that dietary phytochemicals trigger a condition defined hormesis [1, 8, 13, 14]. This process is due to low-doses of bioactive compounds that act as mild stressors to induce adaptive expression of stress-protective genes and enhance resistance to mechanisms that determine ageing. The signal passes through the modulation of kinases, leading to the activation of downstream targets, among which FoxO3A and sirtuins. In this last case, it is to note the known effect of resveratrol, phytochemical contained in the grape, that, downstream, inhibits the NF-kB pathway, with interesting anti-inflammatory effects, although it is not totally clear and contrasting results exist [15]. *In vitro* and *in vivo* evidences suggested that many phytochemicals can affect the expression of numerous genes encoding pro-survival proteins, including antioxidant enzymes, neurotrophic or anti-apoptotic factors [1, 8].

Extra-virgin olive oil (EVOO) is full of these compounds, such as hydroxytyrosol, tyrosol and secoiridoids. It is extracted from olive fruits of *Olea Europea* and its health beneficial effects are well established. An extensive literature demonstrated that they can be attributed to many different substances belonging to the phenolic fraction of EVOO. However, the concentration of these molecules is strongly affected by belonging to a particular *cultivar*, by agronomic and environmental factors, and by the extraction and storage conditions [8, 16, 17].

Interestingly, experimental evidence suggests that hydroxytyrosol is not only effective in removing reactive oxygen species generated by impaired redox balance but it may also be a potent inducer of phase II detoxifying enzymes and an enhancer of mitochondrial biogenesis. Indeed, its effect is mostly observed at the transcriptional level through modulation of the redox-sensitive nuclear factor-E_2-related factor 2 (Nrf2), which regulates gene expression of several phase II detoxifying enzymes and supports the structural and functional integrity of the mitochondria. For instance, in a study performed on rats, hydroxytyrosol supplementation improves neurogenesis and cognitive function through increased activity of the transcription factors FoxO1 and FoxO3, as well as Nrf2, resulting in decreased oxidative stress and increased mitochondrial function [18].

Several health claims for EVOO and its derivatives have been assessed in recent years but only one was authorized in Europe. It relates the impact of olive phenolic compounds on the protection of blood lipids from oxidative stress: "A daily intake of 20 g of olive oil, which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein and tyrosol) provides the expected beneficial effects" [19].

Phytochemicals are also ingredients of many dietary supplements commercially available to prevent or ameliorate specific diseases, including age-related ones. The majority of these products are not substantiated by solid scientific evidences and have not been approved yet by the EFSA and/or FDA. Beyond them, functional foods are widely used, especially in Japan and in USA. Although a universal definition of them does not exist, they are often considered as foods with healthy properties that contain bioactive compounds naturally or added to obtain processed foods.

However, further observational studies and dietary intervention trials in large cohorts of healthy subjects are essential to evaluate whether these foods and compounds can help to prevent age-related disorders.

The series

To discuss the potential relevance of nutrigerontology in the attainment of successful ageing and longevity, three scientific studies and two reviews have been assembled in this series.

As discussed by Passarino et al., on the whole, although the genetic factors account for only 25 % of human lifespan, the knowledge of the genetic basis of longevity may give significant hints on modulating lifestyle, in order to extend health span. That is, a few subjects can attain successful ageing and longevity thanks to a lucky combination of polymorphisms, which allow them to have an efficient metabolism or an efficient response to different types of stress. Most of the others can reach similar results by targeting the same pathways with appropriate lifestyle or interventions to slow ageing. In this context, the importance of epigenetic factors, both as biomarkers of ageing and target of interventions will certainly grow in the forthcoming studies [20].

The aim of the study of Accardi et al., was to analyse the nutraceutical properties of table green olives *Nocellara del Belice*, a traditional Mediterranean food, since little is known about the role of olives as nutraceutics. After the intake of 12 olives a day for 30 days, a significant decrease of malondialdehyde, a molecule related to oxidative stress, was observed. In addition, the level of interleukin-6 (IL-6) underwent a significant reduction, demonstrating how this food could be able to modulate the inflammatory response. Moreover, it was noteworthy the reduction of fat mass with an increase of muscle mass, suggesting a possible effect on long time assumption of table olives on body mass variation [9].

In Barera et al. study, the authors considered the nutraceutical effects of β-glucans, alimentary fibers, added to pasta, in order to produce a functional food. After 30 days of pasta intake, they obtained encouraging results with a significant decrease of low-density lipoprotein cholesterol, IL-6 and advanced glycation end-product levels. In fact, MedDiet is also characterized by a large intake of fibers, which contribute to lowering cholesterol and are positively associated with colon cancer prevention. However, in Mediterranean towns most people do not have a close adherence to MedDiet, hence adding fibers to pasta would achieve the same effects [21].

Data from dietary intervention studies of Carruba et al. underlined some interesting aspects related to mechanisms underpinning both biological and clinical effects of nutrition and specific activities of Mediterranean food components. In particular, the authors provided evidence that MedDiet may regulate oestrogen metabolism in postmenopausal women. In fact, it seems that the formation of potentially harmful genotoxic oestrogen compounds is remarkably reduced by the adoption of a traditional Mediterranean dietary model, while the levels of parent hormone estradiol become slightly increased. So, this result would imply that traditional Mediterranean food reduce the risk of developing breast cancer, while limiting the side effects of oestrogen withdrawal in menopause. Technological innovation and prototypical industrialization of either, processes or products, could be used to obtain traditional Mediterranean food with high health potential and market capacities. Precisely, the production of monocultivar EVOOs revealed that they may have differential activity on cellular and metabolic processes, eventually leading to produce highly characterized EVOOs with a preferential use for the prevention and care of various chronic diseases [22].

The review of Davinelli et al., attempted to summarise recent evidences about phytochemicals as anti-oxidants and anti-inflammatories. In fact, as previous mentioned, they may act as positive modulators of inflammation and oxidation by attenuating pro-inflammatory signalling associated with the redox imbalance that occurs in brain ageing. They also discussed the need to initiate long-term nutritional intervention studies in healthy subjects. In fact, their manuscript highlighted crucial aspects but that require further studies to determine the effective physiological concentrations and to explore the real impact of dietary phytochemicals in preserving brain health before the onset of symptoms leading to cognitive decline and inflammatory neurodegeneration [23].

Discussion

As stated by Kolovou et al. [3] during the last two centuries the average lifespan has increased at a rate of approximately 3 months/year in both sexes. The most important steps in prolonging human lifespan were the decrease of child and maternal deaths, the lowering of infant and juvenile mortality rate due to, respectively, vaccination and treatment of infectious diseases. Moreover, in the last decades, the survival of elderly people improved thanks to secondary and primary prevention of ageing-related diseases and, particularly, coronary heart one [3].

So, all the factors above mentioned contribute to the fact that oldest old people are becoming the population with the fastest growth in Western World. Although the average life expectancy is increasing dramatically, the healthy lifespan is not going at the same pace. Hence, ageing and age-associated diseases are emerging as among the greatest challenges and financial burdens, faced by developed and developing countries [7].

Data from experimental studies in model organisms have consistently shown that both chronic dietary restriction, affecting nutrient signalling pathways, and mutations in nutrient and growth signalling pathways can extend longevity by 30–50 % [6, 7]. Also, they can lower the prevalence of age-related loss of function, including immunosenescence and multiple diseases, such as cancer, cardiovascular disease, and neurodegeneration [6, 7, 24].

In addition, several experimental data clearly demonstrate the hormetic effects of phytochemicals by inducing cellular stress resistance mechanisms [1, 8]. In designing human intervention studies to provide high-quality evidence for their health benefits, the following factors have to be considered: 1) the phytochemicals need to be sufficiently characterised, as well as its optimal physiologic dose, 2) the characteristics of targeted populations including their nutritional status, health condition, and genetic background have to be taken into account, 3) clinically relevant, sensitive, reproducible, and feasible endpoints have to be identified, 4) length of the intervention have to be commonly agreed [24–26]. However, even though experimental data have not always translated to a definitive clinical effect, the antioxidant and anti-inflammatory properties of phytochemicals have been widely accepted.

Conclusion

The new findings presented in the experimental studies of the series give a great achievement for the food and farming industry, especially in Sicily, where local products represent a great potential resource. No approved healthy property and claim exist for them. Therefore, adding such products to the class of "healthy food" could represent a big deal. In the era of many expansive and mysterious longevity elixirs, they could represent a traditional, cheap and accessible "healthy food" to everyone. But more than for the single food, the Accardi's study [9] highlights the importance to analyse local products that are traditional and easy to find. So, in Italy one should find phytochemicals in table green olives, in China in the Goji berries. In fact, the key is not a specific molecule in a specific food but its beneficial effect and the possibility for everyone to benefit.

The interesting effects of nutraceuticals and functional foods could represent a prevention for many age-related diseases, and, if not a total solution, at least a piece of their puzzle.

So, the possibility to create a dietary pattern, based on the combined strategy of the use of both nutraceuticals and functional foods, should permit to create a new therapeutic strategy based not only on a specific bioactive molecule or on a specific food but on a integrated approach that, starting from the local dietary habits, can be led to a "nutrafunctional diet" applicable worldwide.

Additional longitudinal observations on community-based cohorts are needed to confirm these data and investigate the biological mechanisms, including epigenetic ones, through which effects are induced, and to fully explore their therapeutic potential.

Nevertheless, nutrigerontology, putting together branches strictly related to ageing process, as biogerontology, medicine and nutrition, should be the key for achieving successful ageing and longevity.

Competing interest

The authors declare no conflict of interest.

Authors' contributions

CC wrote the paper. All authors edited the paper and approved its final version.

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Author details

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Palermo, Italy. [2]Division of Research and Internationalization, ARNAS-Civico Di Cristina e Benfratelli, Palermo, Italy. [3]Department of Medicine and Health Sciences, School of Medicine, University of Molise, Campobasso 86100, Italy. [4]Department of Biology, Ecology and Earth Science, University of Calabria, Rende (CS) 87036, Italy. [5]Department of Biological Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy and Institute of biomedicine and molecular immunology "Alberto Monroy" CNR, Palermo, Italy.

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XI. Discussion and conclusions

The present thesis confirms the widely discussed role of the MedDiet and functional foods on health.

As it was demonstrated, a close adherence to a Mediterranean-style diet permits one to avoid some cardiovascular disorders and cancers and is responsible for the high rate of long-living individuals scattered throughout the world. In 2011, five places: Okinawa in Japan, Sardinia in Italy, Ikaria in Greece, Loma Linda in California, and Nicoya in Costa Rica, have been recognized to have a very high prevalence of healthy octogenarians. These countries have been identified as “Blue-Zones”. The term given to these areas defines them as limited regions whose population shares a common lifestyle and environment, and whose exceptional longevity has been accurately verified (*Buetner, 2011*). Among the lifestyle habits, the adopted nutritional patterns are similar to that of the Mediterranean, since their diets are characterized by a high/quality consumption of fruit, wild plants and vegetables, and low consumption of meat products. Other common features to these populations are high levels of daily physical activity, positive attitudes, and work hours spaced out

with periods of calm and mid-day siesta (*Chrysohoou et al., 2011*; *Chrysohoou and Stefanadis, 2013*).

Using nutritional interventions as means to study the effects of Mediterranean functional food on humans, we showed that some Mediterranean plant-foods or derivatives, like olives, pasta with β-glucans, and pasta with OFI cladode extracts, have antioxidant and anti-inflammatory effects on healthy or non-healthy subjects. We also showed that an over intake of fibers through processed functional foods can act on gut hormones, exhibiting promising responses on glucose metabolism and on microbiota modification.

We mainly recruited young and middle age people to study, at the best of our ability, the inter/intra-individual variability, and to evaluate the role of healthy eating on healthy ageing. We also demonstrated that several molecular pathways, like NPS ones, affecting metabolism, growth, inflammation, and epigenetic modifications, which alter the rate of ageing and incidence of age-related diseases, can be modulate by nutrition. The well-known are insulin/IGF-1, mTOR and sirtuins pathways. Their association with ageing and longevity processes was widely approved (*Fontana et al., 2010*; *Wątroba and Szukiewicz, 2016*) (see **V**.). In particular, it was seen that

the downregulation of IGF-1 and mTOR cascade or the up-regulation of sirtuins one, through a low GI and low animal protein intake, in synergy with bioactive compounds, can extend lifespan in various model organisms, including mammals (*Ziv and Hu, 2011*). As previously reported, these effects were also obtained by the presence of specific single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in NSPs, such as IGF-1 receptor or FOXO 3A. For example, some FOXO3A SNPs were associated with longevity in different populations. Certain variants were found with higher frequency in centenarians, highlighting their role in SA probably due to an increased activity of FOXO3A and the consequent interaction with sirtuins (*Di Bona et al., 2014*; *Kenyon,* 2005; Suh *Y et al., 2008*).

In the same way, it was suggested that the beneficial effect of CR on lifespan was linked to decreased circulating IGF-1 levels and, consequently, to the activation of FOXO3A and the inhibition of mTOR Complex 1. On the other hand, the downregulation of insulin/IGF-1 signaling pathway inhibits the NF-κB signalling, a known pathway involved in immune inflammatory mechanisms, with interesting anti-inflammatory effects (*Gilmore and Wolenski, 2012*; *Newton and Dixit, 2012*).

A recent review underlines that one of the best human models of CR without malnutrition is the Okinawa diet. Okinawans consume approximately 17% fewer calories than the average adult in Japan. The Okinawa diet is also reported to be lower in protein and rich in fresh vegetables and fruits, sweet potatoes and soya (*Pallauf et al., 2013, Willcox et al., 2007, 2014).*

Polyphenols (such as resveratrol in grapes and red wine, and secoiridoids in olives and EVOO) can also regulate NF-κB, reducing the expression of inflammatory cytokines and activating sirtuins (in particular SIRT1) pathway (*Martín-Peláez et al., 2013*). In this regard, we demonstrated that table green olives *Nocellara del Belice* have anti-inflammatory effects on healthy subjects because the levels of IL-6 were significantly decreased (see **VI.**). Recently, Longo also used green olives in FMD, probably for their positive effects on health.

In addition, it must be stressed that several insights evidenced that the induction of a mild stress is a promising strategy to slow down ageing and prevent or postpone the onset of age-related disabilities. This induces hormesis (*Davinelli et al, 2012*; *Rattan, 2012*). Its role in ageing has already been examined by Rattan (*Rattan,* 2008) but it also has relevance to

explain the anti-ageing and life-extending actions of CR in long-lived species (*Masaro, 2007*).

Hormesis is a process triggered by exogenous agents or environmental conditions and it is likely identified as an integral part of the normal physiological function of cells and organisms. Hormetins are molecules, like the phytochemicals of fruits and vegetables produced by plants as protection against microorganisms, which can activate the hermetic process. Polyphenols belong to this category. These molecules induce cellular stress response and, subsequently, adaptive stress resistance (*Mattson, 2008*). Stress resistance involves molecular adaptations and induces many of the nutrient-sensing longevity pathways like Nrf2. It is a central regulator of the adaptive response to oxidative stress but very few studies have investigated its role in the modulation of ageing and longevity (*Scapagnini et al., 2011*). However, it was seen that when Nrf2 is translocated into the nucleus, it binds Nrf2-antioxidant response element (ARE), determining the activation of genes that encode antioxidant enzymes, resulting also in endothelial protective effects. Molecules that act in this way are, for example, is othiocyanates in Brassicaceae like broccoli, cabbage, and cauliflower, (*Accardi et al., 2016a*;

Ungvari et al., 2010). Moreover, interesting experimental evidence suggests that also hydroxytyrosol has positive effect on the modulation of the transcription of Nrf2. For instance, in a study performed on rats, hydroxytyrosol supplementation improves neurogenesis and cognitive function through increased activity of the transcription factors FOXO1 and FOXO3, while Nrf2 results in decreased oxidative stress and increased mitochondrial function (*Zheng et al., 2015*).

In our research, we showed that AOP, a semisynthetic peracetylated oleuropein derivative, works in a dose-dependent manner, scavenging the ROS in PBMCs medium, in presence of hydrogen peroxide, after 24 hours of treatment, probably activating AREs. This confirmed its antioxidant activity (**Figure 12**, **13**, **14**). Indeed, the antioxidant compounds are able to modulate the production of free radicals. The role of free radicals as mediators of tissue damage is recognized in many pathophysiological processes such as inflammaging, atherosclerosis, ischemia, tumors, and other age-related disabilities (*Kehrer JP and Klotz, 2015*). In this regard, we showed that a functional food, processed with OFI cladode extracts, has antioxidant power. It improves some healthy parameters, causing PON and GSH, antioxidant molecules,

increase. Also green olives have demonstrated antioxidant activity, as a matter of fact that MAD, a marker of lipid oxidation, significantly decreases (see **VI.**).

Moreover, although the beneficial effects of Mediterranean nutraceuticals on the cardiovascular system have long been known, only recently have scientists focused their attention on the role of nutraceuticals and, in general, of "nutritional therapy" on endothelial function. In particular, the possibility to improve endothelial state and to modulate inflammation is achievable by modulating the functional activity of endothelial progenitor cells (EPCs). We showed that the MedDiet is able to decrease ROS production and, so, to reduce endothelial damage, by determining lower apoptosis rate in endothelial cells. Moreover, *in vitro* studies demonstrated that antioxidant bioactive compounds from olive oil, by decreasing oxidative stress by the modulation of Nrf2 pathway, restore the EPCs function and reduce the number of senescent cells (*Accardi et al., 2016a*).

According to several interventional studies, like PREDIMED, the largest randomized primary prevention trial showing that an intervention to promote a Mediterranean diet is beneficial against the CVDs (http://www.predimed.es), we also

showed that the functional food pasta with *Opuntia* has beneficial effects on TC and on abdominal and thoracic waists (see **Table 4**). This is in agreement with the literature that evidences that the supplementation with OFI generates effects on body fat and TC (*Onakpoya et al. 2014*). In addition, at the end of the table green olives intervention, we observed a reduction in fat mass with an increase in muscle mass probably linked to the activity of CLA. Also *Lehnen et al*. demonstrated that CLA supplementation could also increase lipolysis and reduce the accumulation of fatty acids on the adipose tissue; the putative mechanisms involved may be its action in reducing the lipase lipoprotein activity (*Lehnen et al., 2015*). Although other studies made in humans have shown some benefits of CLA supplementation for weight loss, the results are still discordant. In line with these data, our group showed that pasta enriched with β-glucans is able to implicate a significant decrease of LDL-cholesterol, IL-6 and AGEs levels, confirming the promising role of fibers intake in Mediterranean dietary pattern (*Barera et al., 2016*). If further analyses will confirm this, pasta with β-glucans may be used in the prevention of diabetic disease, for its role on glucose metabolism and gut hormones linked to sense of satiety. It

must be stressed that the effects of regular intake of higher amounts of β-glucans on gut hormones in humans, that concur to regulate appetite/satiety, have not been defined.

Another aspect that we investigated during our nutritional interventions was the possible prebiotic effect of functional foods and the consequently microbiota modulation. The relationship between diet, microbiota and, in turn, health and longevity, is yet obscure. However, a large number of recent studies have investigated the importance of diet, among lifestyle factors, that influence the composition of the gut microbiota (*Sonnenburg and Bäckhed, 201*6).

The human microbiota is the set of the 10-100 trillion symbiotic microbial cells present in gastro-intestinal tract (GIT) of each person, primarily bacteria; the human microbiome consists of all microbes genes and it can be considered a counterpart to human genome (*Turnbaugh et al., 2007*). Such compounds, considered as prebiotics, defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacterial species in the colon, thus improving host health (*Gibson and Roberfroid, 1995*). Many of the health-promoting effects attributed to prebiotic substrates

are due to their suitability to be fermented by the colonic microbiota producing short-chain fatty acids (SCFA).

The correlation between diet and microbiota was analysed both in mice and in humans but more studies are necessary to evaluate the prebiotic effect of foods and food constituents, especially fruits, vegetables, and phytochemicals. However, it was ascertained that specific dietary pattern is associated with the increase or the decrease of specific bacterial population (*Clarke et al., 2012*; *Graf et al., 2015*).

Nowadays, several research groups have also investigated the contribution of gut microbial communities to old people and their role on healthy ageing because of it has a strong impact on human metabolism and immunology. The microbiota composition is not stable over the whole life-time of an individual. It is affected by ageing. Age-related gut microbiota changes are mainly associated with immunosenescence and inflammageing, and with changes in diet and in host immunity reactivity. For example, the prolonged retention time leads to an increase in bacterial protein fermentation and consequently putrefactive processes in the gut with a greater susceptibility to inflammatory diseases (*Woodmansey, 2007*). Changes with age in specific bacterial

genera and species have been identified, with a considerable inter-individual variation that continues into old age. Many studies have demonstrated a decline, with the increased age, in counts of *Bacteroides* and *Bifidobacteria*, both in the total numbers and in the species diversity that is amplified consequently to the antibiotic therapy (*Biagi et al., 2013*; *Jeffery et al., 2013*).

In our pilot studies, after the consumption of 12 table green olives/day, in middle-aged people we did not observe statistically significant differences in the *Lactobacilli* amount in feces, although a positive trend towards an increase was observed. It is plausible that a long-term dietary intervention could be stronger in terms of variation of this analysed parameter. In the second study, we noted that the dietary intake of foods rich in oligosaccharides had beneficial effects on intestinal functions. The prebiotic effect of mucilage and pectic-derived oligosaccharides from *Opuntia* have yet been reported in literature, and resulted in relevant increase in *Lactobacilli* and *Bifidobacteria* populations (*Guevara-Arauza et al.,* 2012). In contrast, our results showed that dietary supplementation with OFI cladode extracts, embedded in pasta, resulted in an overall decrease in *Enterobacteriaceae* and

Lactobacilli populations, where the latter was more heavily affected. This could suggest a possible negative effect of the *Opuntia* dietary supplementation on microbiota, probably due to the high anti-microbiological property of OFI cladode extracts or to the bacterial competition for adhesion to the gut mucosa. Our data about bacterial reduction remain difficult to explain, even considering that neither "trapping" effect nor specific attachment of *Lactobacilli* to dietary fibers (which could explain a decreased number due to reduced mucosal colonization) have been reported to date. Moreover, the different route of intake has to be highlighted, since the inclusion of *Opuntia* into wheat pasta (consisting mainly in a protein and starch matrix) might alter the availability of different components and, in turn, the overall effect. On the contrary, a positive promising link between fibers-food intake and microbiota was seen for β-glucans consumption. Indeed, in our pilot study we showed that, at the end of the intervention, *Bifidobacterium Longum* increased in 80% of subjects analyzed (8/10), from 2 to 36-fold greater than the baseline, while *Enterococcus faecium* showed a decrease in 90% (9/10) (see **VII.**).

In conclusion, the healthcare costs in many countries are very high because of the increased number of unhealthy populations and the consequent increase of age-related disabilities. However, experiments in laboratory organisms have shown that ageing is not an immutable process. Indeed, interventions to slow or postpone ageing and to increase the active life expectancy are available. Pharmacological therapy is one of these. However, our data strengthen the possibility that nutrition could be the alternative to pharmacological approaches to improve SA.

As we discussed, some dietary interventions should be pursued. A feasible diet that reduces the risk of ageing-related diseases, promoting health ageing and longevity, should be one that delivers refined carbohydrates and amino acids in a pro-ageing way, in activating or reducing NSPs. It is not necessary to determine the ideal composition of this diet, because it does already exist. It is the traditional “poor” MedDiet, which more than nutrients or single foods, can act on ageing. In fact, the key for anti-ageing therapies is not found in a specific molecule in a specific food but it is found in the beneficial dietary pattern from which everyone can benefit.

Nutritional education could be the way to improve health and quality of life of mid and old people, increasing the potential relevance of “dietary therapy”. In this regard, we believe that nutrigerontology, putting together branches strictly related to ageing process, as biogerontology, medicine and nutrition, should be the key for achieving SA and longevity.

Furthermore, the new findings presented in the experimental studies of this thesis could present a great opportunity for the food and farming industry, especially in Sicily, where local products like EVOO, green olives, barley and OFI, represent a great potential resource.

In the era of many expensive and mysterious longevity elixirs, these resources could represent traditional, cheap, and accessible “healthy foods” for everyone. However, it is important to highlight that the interesting effects of nutraceuticals and functional foods could represent a prevention for many age-related diseases, and not a solution.

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