UNIVERSITÀ DEGLI STUDI DI PALERMO

DIPARTIMENTO DI BIOPATOLOGIA
E BIOTECNOLOGIE MEDICHE
SEZIONE DI PATOLOGIA GENERALE

dibmed

Dottorato di ricerca in Medicina Molecolare

XXVI Ciclo

SSD MED/04

Fasting and "Fasting mimicking diet" (FMD) an effective intervention to promote longevity and health span

Candidata: Claudia Virruso

Tutor: Prof.ssa Giuseppina Candore

Coordinatore: Prof. Calogero Caruso

TABLE OF CONTENTS

ABSTRACT OF PAPERS PRODUCED DURING PhD COURSE AND RELEVANT TO THIS THESIS

1. Di Bona D, Accardi G, **Virruso C**, Candore G, Caruso C. Association Between Genetic Variations In The Insulin/Insulin-Like Growth Factor (Igf-1) Signaling Pathway And Longevity: A Systematic Review And Meta-Analysis. Curr Vasc Pharmacol. 2014;12(5):674-81. Review.

Abstract

Some studies have shown that polymorphisms in the insulin growth factor-1 (IGF-1) signaling pathway genes could influence human longevity. However, the results of different studies are often inconsistent. Our aim was to investigate by systematic review and meta-analysis the association of the common polymorphisms defining the genetic variability of the IGF-1 signaling pathway associated with human longevity. Eleven studies investigating the association between the polymorphisms in the IGF-1 signaling pathway genes (IGF-1, IGF-1 receptor (IGF-1R), Forkhead box O3A (FOXO3A) and Silent mating type information regulation 1 (SIRT1) and longevity were found and analyzed. The model-free approach was applied to meta-analyze these studies. No association was reported between the single nucleotide polymorphisms (SNPs) of IGF-1 and longevity in the available study. The meta-analysis of available data from four studies, showed a significant association with the IGF-1R polymorphism rs2229765, suggesting that subjects with the A-bearing genotype have greater chance of longevity. Concerning the five studies on FOXO3A SNPs, for the rs2764264 significant association with longevity was observed for C allele when only males were included in the analysis. Statistically significant results were obtained for other SNPs as well, i.e. rs2802292 (G allele), rs9400239 and rs479744 (T and A alleles, respectively). For rs9400239 the association was observed in male long lived with a lower odds ratio than in centenarians while in rs479744 it was highlighted a significant association in centenarians. Concerning SIRT1, no association between the SNPs under study and longevity was observed in the only available report. Current findings suggest that both IGF-1R and FOXO3A polymorphisms could be associated with longevity. The high degree of between-study heterogeneity and the low number of available studies underline the

need for further methodologically adequate analyses to confirm these evidences.

2. Caruso C, Candore G, Accardi G, **Virruso C**, Di Bona D. Association of Klotho polymorphisms with healthy ageing: a systematic review and meta-analysis. Rejuvenation Res. 2014 Apr;17(2):212-6. doi: 10.1089/rej.2013.1523. Epub 2014 Apr 11. Review.
Abstract
Nowadays is clearly evident that genetic background constitutes integral part of ageing and longevity. Many studies on long lived people have been conducted emphasizing the role of certain genes in long life. Classic case-control studies, genome wide association studies and high throughput sequencing have permitted to identify a variety of genetic variants seemingly associated with longevity. Over the years, ageing research has focused on insulin/IGF-1 signaling pathway because of its evolutionary conserved correlation with life-span extension in model animals. Indeed, many single nucleotide polymorphisms (SNPs), associated with longevity were identified in genes encoding proteins that take part in this metabolic pathway. Closely related to this pathway is the Klotho gene. It encodes a type-I membrane protein expressed in two forms, membrane and secreted. The last form acts suppressing oxidative stress and growth factor signaling and regulating ion channels and transporters. In particular, its over-expression seems to be able to suppress insulin/IGF-1 signaling extending life span. Thus, our aim was to put together the results showed in literature concerning the association between the functional variant of KLOTHO "KL-VS" stretch that contains six polymorphisms in linkage disequilibrium and successful ageing to quantify the possible effect of the variants. The results of our systematic review indicate that Klotho KL-VS variant is associated with healthy ageing.

3. Accardi G, **Virruso C**, Balistreri CR, Emanuele F, Licastro F, Monastero R, Porcellini E, Vasto S, Verga S, Caruso C, Candore G. SHIP2: a "NEW" insulin pathway target for ageing research. Rejuvenation Res. 2014 Apr;17(2):221-5. doi: 10.1089/rej.2013.1541.
Abstract

Strong evidence suggests that systemic inflammation and central adiposity contribute to and perpetuate metabolic syndrome. All of these alterations predispose individuals to type 2 diabetes mellitus (T2DM), cardiovascular disease, as well as Alzheimer's disease (AD), all characterized by chronic inflammatory status. On the other hand, extensive abnormalities in insulin and insulin growth factor(IGF)-I and IGF-II signaling mechanisms in brains with AD have been demonstrated, hence suggesting that AD could be a third form of diabetes. The Src homology domain-containing inositol 5-phosphatase(SHIP)2, has an important role in insulin pathway because its over-expression causes impairment of insulin/IGF-1 signaling. Since some single nucleotide polymorphisms (SNP) of the gene encoding SHIP2, were significantly associated in T2DM patients with metabolic syndrome and some related conditions, we decided to conduct a case-control study on this gene, analyzing AD and T2DM subjects as cases and young, old and centenarians as controls. Our results suggest a putative correlation between the rs144989913 SNP and ageing, both successful and unsuccessful, rather than age-related diseases. Since this SNP is an insertion/deletion of 28 base pairs, it might cause an alteration in SHIP2 expression. It is noteworthy that SHIP2 has been demonstrated to be a potent negative regulator of insulin signaling and insulin sensitivity. Many studies demonstrated the association of insulin/IGF1 pathway with ageing and longevity, so it is tempting to speculate that the found association with SHIP2 and ageing might depend on its effect on insulin/IGF-1 pathway.

4. **Virruso C**, Accardi G, Colonna Romano G, Candore G, Vasto S, Caruso C. Nutraceutical properties of extravirgin olive oil: a natural remedy for age-related disease? Rejuvenation Res. 2014 Apr;17(2):217-20. doi: 10.1089/rej.2013.1532. Abstract
   The health benefits of the Mediterranean Diet can be largely ascribed to the nutraceutical properties of extra-virgin olive oil (EVOO). Monounsaturated fatty acids and various phenolic compounds such as oleocanthal, oleuropein, hydroxytyrosol and tyrosol are the main nutraceutical substances of EVOO. These substances have been suggested to have the ability to modulate ageing- associated processes. In experimental models, it was shown that EVOO with high concentration of poliphenols has anti-inflammatory and antioxidant properties. Indeed, it was observed that hydroxytyrosol, as well as

oleocanthal, inhibit the cyclooxygenases (COX-1 and 2), responsible for prostaglandin production; oleuropein is a radical scavenger that blocks the low-density lipoproteins oxidation. Due to the relevance of the olive oil in the economy of Sicily, our group has been funded to assess the nutraceutical properties of different kinds of olive oil. Indeed, the aim of the study is to evaluate effects of EVOOs, with low and high polyphenols content, on the immuno- inflammatory and oxidative stress responses in young and old people. Further objective of our group is to evaluate effects of EVOO, with low and high polyphenols content, on the expression of genes encoding proteins that take part in Insulin/Insulin-like growth factor-1 signaling pathway involved in longevity. The results of the study will be useful to produce olive oil enriched in nutraceutical properties, likely helpful in the prevention of age-related diseases.

5. Caruso C, Accardi G, **Virruso C**, Candore G. Sex, gender and immunosenescence: a key to understand the different lifespan between men and women? Immun Ageing. 2013;10:20.
   Excerpta
   Gender and sex are known to be associated with longevity. While males are usually stronger, females live longer. In the Western world, the life expectancy of individual born between 2005 and 2010 is 80.4 for women and 73.4 for men. Potential factors have been examined to explain this disagreement. It is possible distinguish advantage in longevity related to biological traits and factors related to socio-cultural characteristics of the population. Males and females have different behavioural tendencies, social responsibilities and expectation. So, differences in mortality between men and women can be not only a matter of sex that refers to biological differences, but also a matter of “socially constructed sex”, i.e. gender. One of the main interaction between gender and longevity is linked to the kind of job. Indeed, in the to-day elderly, professional exposure to stressors was stronger in males rather than in females.

LIST OF ABBREVIATION

AD Alzheimer's diseases

CO Centenarian offspring

CVD Cardiovascular disease

EVOO Extra virgin olive oil

FOXO Forkhead box O

FMD Fasting Mimicking Diet

GSK3 Glycogen synthase kinase 3

GPCRs G-protein-coupled receptors

HFD High Fat Diet

IF Intermittent fasting

IGF Insulin-like growth factor

IGF-1R Insulin growth factor-1 receptor

IkB inhibitor of kB

IkK inhibitor of kB kinase

IR Insulin resistance

IRS Insulin responsive substrate

LLI Long lived individuals

LPS Lipopolisaccaride

MUFA Monounsatured fatty acid

MD Mediterranean diet

MS Metabolic syndrome

OR Odd ratio

PI3K Phosphoatidyl inositol 3-kinase

PIP2 Phosphoatidyl inositol 2-phosphate

PIP3 Phosphoatidyl inositol 3-phosphate

RAS Rat sarcoma protein

SHIP2 Src homology domain-containing inositol 5-phosphatase 2

SIRT1 Silent mating type information regulation 1

STS Short-Time-Starvation

SNP Single nucleotide polymorphism

T2DM Type 2 diabetes mellitus

TF Transcription factor

CHAPTER 1

FASTING AND HEALTHY LIFESPAN FROM PROKARYOTES TO HUMANS: FOCUS ON INSULIN/IGF-1 PATHWAY.

1.1 INTRODUCTION

Since Hippocrates and Plutarch was quoted as asserting "*instead of using medicine, rather fast a day*", fasting has been practiced as a treatment of many illnesses, but only recently several studies have shown the its beneficial effects in adaptive cellular responses that reduce oxidative damage and inflammation, optimize energy metabolism and enhance cellular protection.

Many religious groups incorporate periods of fasting into their rituals, millions of Muslims refrain from eating or drinking from sunrise (Sahur) to sunset (Iftar) during the holy month of Ramadan, which lasts between 28 and 30 days. Christians, Jews, Buddhists and Hindus traditionally fast on designated days of the week or calendar year. In many clinics, patients are now monitored by physicians while undergoing water only or very low calorie (less than 200 kcal/day) fasting periods lasting from 1 week or longer for weight management, and for disease prevention and treatment. Emerging discoveries from studies of animal models and humans suggest that the intermittent abstinence by food and caloric beverages of as little as 12 hr,

intermittent fasting (IF) or 48-120 hr, prolonged fasting (PF), improves health effects and protects from diseases processes. In rodents, fasting for 24 hr every other day or twice weekly extends lifespan up to 30%, independent of both total food intake and weight loss (*Mattson et al., 2014*) and promotes protection against diabetes, cancer, heart disease, and neuro-degeneration (*Longo and Mattson, 2014*). In humans, IF and less-severe regimens (e.g., consumption of approximately 500 kcal/day for 2 days a week) have beneficial effects on insulin, glucose, C-reactive protein, and blood pressure (*Harvie et al., 2011*). PF cycles lasting 2 or more days, but separated by at least a week of a normal diet, are emerging as a highly effective strategy to protect normal cells and organs from a variety of toxins and toxic conditions. Raffaghello et al. found that fasting for 48 or more hours protects mice and/or normal cells, but not cancer cells from various chemotherapy drugs (*Raffaghello et al., 2008*), while increasing the death of many cancer cells type. This effect seems to be dependent in part on the reduction of the circulating IGF-1 and glucose levels accompanied by authofagy (*Lee et al., 2012*). Recently, a study has shown that, in mice, multiple cycles of fasting modulate hematopoietic stem cells protection, self- renewal and regeneration via IGF-1 or PKA inhibition (*Cheng et al., 2014*). Moreover, short-term fasting (1–3 days) has been shown to protect rodents against the damage induced by ischemia-reperfusion of the liver and kidney, by improving insulin sensitivity, reducing expression of markers of inflammation and insulin/IGF-1 signaling, and increasing cytoprotective gene expression (*Hine et., al 2015*). Others have

reported on the role of PF in causing major decreases in liver and body mass in rats (*Wasselin et al., 2014*).

The fascinating and potent effect of different kinds of fasting could be explained trough the molecular mechanisms of the nutrient-sensing pathways connecting nutrition to the ageing process. Interestingly, most mutations, capable to extend life-span in a wide range of organisms affect proteins that, directly or indirectly, participate in nutrient-sensing pathways among them one of the most important is the insulin/insulin –like growth factor (IGF-1 pathway). The explanation is that these mutations mimic calorie restriction (CR, a dietary regimen based on low calorie intake, usually 30% lower than normal without malnutrition), a process well known to be involved in life-span extension. Indeed, literature data obtained using model organisms, from prokaryotes to mammals, highlight the role of these pathways in life-span modulation (*Longo et al 2003*).

The remarkable effects of the CR on aging and diseases in mice and rats are often viewed as responses evolved in mammals to adapt to periods of limited availability of food. In these models, reduced nutrient signaling is associated with decreased levels of free IGF- 1 (*Fontana and Klein, 2007; Fontana et al., 2010; Masoro, 2005; Weindruch and Walford, 1988*). The cellular and molecular mechanisms responsible for the protective effects of CR have likely evolved billions of years earlier in prokaryotes endeavoring to survive in an environment mostly or completely deprived of energy sources while prevent from age-dependent damage that could compromise fitness. In fact, the bacterium *Escherichia coli* switched from LB, a nutritionally peptide rich medium containing glucose, to a calorie-free

medium survives 4 times longer than the same strain inoculated in LB (*Longo and Mattson; 2014*); this effect reversed by the addition of nutrients but not acetate, a carbon source associated with a starvation condition; inasmuch as, mutants as LipA and LpdA, that genetically promote aspects of hypoxic metabolism extend the stationary phase survival in an hypoxia-inducible factor (ArcA) and acetate- dependent manner (*Gonidakis et a.l, 2010*). The effect of rich medium but not acetate in reducing longevity raises the possibility that a ketone body-like carbon source such as acetate may be part of an "alternate metabolic program" that evolved billions of years ago in microorganisms and that now allows mammals to survive during periods of food deprivation by obtaining much of the energy by catabolizing fatty acids and ketone bodies including acetoacetate and β-hydroxybutyrate (*Cahill, 2006*).

In the yeast *S. cerevisiae*, fasting extends 2-fold chronological lifespan and increases the resistance to multiple stresses (*Longo et al.,1997; Longo et al., 2012*). The mechanisms of nutrients and glucose deprivation leading to the lifespan extension involve the downregulation of two nutrient-sensing pathways, the amino-acids response Tor -S6K (Sch9) and the glucose responsive Ras-adenylate cyclase-PKA pathway resulting in the activation of the serine/threonine kinase Rim15, a key enzyme coordinating the protective responses (*Fontana et al., 2010*). The serine/threonine kinase Rim15 and the downstream stress resistance transcription factors Msn2/4 and Gis1 are required for chronological life span extension in mutants with defects in Ras/cAMP/PKA or Tor/Sch9 signaling as well as in calorie restricted cells (*Wei et al., 2008*).

Notably, when switched to food deprivation conditions, both bacteria and yeast enter a hypometabolic mode that allows them to minimize the use of reserve carbon sources and can also accumulate high levels of the ketone body-like acetic acid (*Longo and Mattson, 2014*). Another major model organism in which fasting extends lifespan is the nematode *C. elegans*. Food deprivation conditions achieved by feeding worms little or no bacteria, lead to a major increase in lifespan (*Kaeberlein et al., 2006; Lee et al., 2006*), which requires AMPK as well as the stress resistance transcription factor DAF-16, similarly to the role of transcription factors Msn2/4 and Gis1 in yeast and FOXOs in flies and mammals (*Greer et al., 2007*). Intermittent food deprivation also extends lifespan in *C. elegans* by a mechanism involving the small GTPase RHEB-1 (*Honjoh et al., 2009*). In flies, most studies indicate that intermittent food deprivation does not affect lifespan (*Grandison et al., 2009*). However, food reduction or food dilution have been consistently shown to extend Drosophila longevity (*Piper and Partridge, 2007*) suggesting that flies can benefit from dietary restriction but may be sensitive to even short starvation periods. Together these results indicate that food deprivation can result in pro-longevity effects in a wide variety of organisms, but also underline that different organisms have different responses to fasting (*Longo and Mattson, 2014*).

In humans, 12 to 24 hours of fasting typically results in a 20% decrease in serum glucose and depletion of the hepatic glycogen, accompanied by a switch to a metabolic mode in which non-hepatic glucose, fat-derived ketone bodies and free fatty acids are used as energy sources. In fact, during prolonged periods of fasting, the brain

and other organs utilize ketone bodies in a process termed ketolysis, in which acetoacetic acid and 3-β hydroxybutyrate are converted into acetoacetyl-CoA and then acetyl-CoA. The ketone bodies are produced in hepatocytes from the acetyl-CoA generated from β oxidation of fatty acids released into the bloodstream by adipocytes, and also by the conversion of ketogenic amino acids (*Longo and Mattson 2014*). These metabolic adaptations to fasting in mammals are reminiscent of those described earlier for *E. coli* and yeast, in which acetic acid accumulates in response to food deprivation (*Gonidakis et al., 2010; Longo et al., 2012*).

1.2 Genetic variants in Insulin/ IGF-1 pathway and prolonged lifespan in humans

Many genetic mutations, in particular single nucleotide polymorphisms (SNPs), extend lifespan by acting on Insulin/IGF-1 pathway. IGF-1, IGF-1 receptor (IGF-1R), Forkhead box O (FOXO) 3A, Silent mating type information regulation 1 (SIRT1) and KLOTHO, all molecules involved directly or indirectly in the Insulin/IGF-1 signaling (IIS) are under reflectors for the association of their SNPs with ageing and longevity. In human beings, ageing is associated with lower IGF-1 circulating levels (*Bartke, 2005*), and in longevous people IGF-1R has been correlated with modulation of human lifespan through the attenuation of IGF-1 signaling (*Suh et al 2008*). Both IGF-1 and IGF-1R polymorphisms theoretically modulating the IGF-1 pathway have been studied for their correlation with longevity, but evidences are not conclusive (*Suh et al 2008; Xie et al 2008; Bonafè et al 2003; Albani et al 2009; Barbieri et al 2012*).
The Insulin/IGF-1 pathway activates the transcription factor (TF) FOXO3A which has also been extensively studied for its role in longevity. FOXO3A gene, belongs to the family and encodes a TF with a typical domain of this family, the forkhead box, a conserved DNA-binding domain. It is one of the orthologues of *daf-16* in *C. elegans*, a TF involved in stress resistance and longevity (*Gems et al 2003; Kenyon 2005*). In addition, FOXO3A interacts with sirtuins, a family of histone deacetylase enzymes, with a controversial role as anti-ageing molecules in model organisms. SIRT1, one of the seven human sirtuin isoforms, SIRT1-SIRT7, deacetylates FOXO3A

modulating its response to oxidative stress (*Brunet 2004*). On the basis of findings from experimental models, some human studies south to demonstrate an association between specific SNPs involved in modulation of Insulin/IGF-1 and longevity. However, the sample size of the most of the studies is inadequate and the results are often inconsistent.

The gene Klotho, aptly-named after one of the Greek goddesses Fates, believed by ancients to spin the thread of life, encodes a type-I membrane protein expresses in two forms, membrane and secreted. It was discovered about fifteen years ago, as a gene which, if knocked out in mice, leads to a syndrome resembling ageing, including short lifespan, while its overespression represses the intracellular response triggered by both insulin and IGF-I, leads to longevity extension ageing and extends lifespan (Kuro-o 2009, Kuroso 2005). On these basis, some studies demonstrated an association between the common functional "KL-VS" variant in the *KLOTHO* (*KL*) gene and longevity in humans. This variant is a stretch that contains six polimorphisms in linkage disequilibrium. However, conflicting results of the association between “KL-VS” and both ageing and longevity exist (*Arking et al 2002; Novelli et al 2008; Invidia et al 2010; Majumdar et al 2010*).

Another protein that has an important role in the insulin pathway thus presumably in ageing and age-related diseases is SHIP2, the Src homology domain-containing inositol 5-phosphatase. The protein SHIP2, encoded by the gene inositol polyphosphate phosphatase-like 1 (INPPL1), catalyzes the degradation of lipid secondary messenger PIP3 to produce phosphoatidil inositol 2 phosphate (PIP2). Thus, SHIP2 is an antagonist of PI3K. Because the PI3K pathway plays a

key role in the biological effects of the insulin, the attenuation of the PI3K mediated insulin signaling pathway could be associated with insulin resistance (IR) in T2DM and with neuropathology of AD (*Steen et al 2005; Frisardi et al 2010*). Many studies underline the role of SHIP2 as probable negative regulator of insulin signaling (*Ferreira et al 2010; Porte et al 2005; Plum et al 2005; Wozniak et al 1993*). A study conducted by Kaisaki et al in T2DM subjects demonstrated a significant association between SNPs of INPPL1 (rs2276047, rs9886 and rs144989913 and MS or correlated correlated features (*Kaiasaki et al 2004*).

1.3 AIM 1 OF THE THESIS

How discussed previously, fasting and the reduction of nutrient sensing pathways signaling increase the lifespan in several model organisms. For example, in rodent, fasting for 24 hr every other day or twice weekly extends lifespan up to 30%, independent of both total food intake and weight loss (*Mattson et al., 2014*) and calorie restriction without malnutrition, reduces insulin/IGF-1 signaling, increasing maximal lifespan up to 50%. Indeed, in model animals, long term calorie restriction, reduces metabolic factors associated with some age-related diseases: oxidative stress, sex hormones and insulin levels, adiposity and inflammation (*Longo et al., 2010*). In human beings, ageing is associated with lower IGF-1 circulating levels (*Bartke, 2005*), and in longevous people IGF-1R has been correlated with through the attenuation of IGF-1 signaling (*Suh et al., 2008*).

Thus, during the first part of my PhD, the aim was to investigate the mechanisms involved in modulation of human lifespan focusing the attention on the role of the insulin/IGF-1 pathway. For this purpose, we explored the literature to summarize the existing data up to date and identified through meta-analysis some genetic variants involved in the lifespan modulation. The genes considered in our studies encoding proteins that take part in the insulin/IGF-1 pathway were: IGF-1, IGF-1R, FOXO3A, SIRT1 and KLOTHO. Moreover, we conducted a case- control study to verify the association of two SNPs of SHIP2 to two age-related diseases, T2DM and AD.

In the following sections we discuss the analysis mentioned above.

1.4 ASSOCIATION BETWEEN GENETIC VARIATION IN THE INSULIN/INSULIN-LIKE GROWTH FACTOR (IGF-1) SIGNALING PATHWAY AND LONGEVITY: A SISTEMATIC REVIEW AND META-ANALYSIS

Send Orders for Reprints to reprints@benthamscience.net

674 *Current Vascular Pharmacology*, 2014, *12*, 674-681

Association between Genetic Variations in the Insulin/Insulin-Like Growth Factor (Igf-1) Signaling Pathway and Longevity: A Systematic Review and Meta-Analysis

Danilo Di Bona[1,2,*], Giulia Accardi[1], Claudia Virruso[1], Giuseppina Candore[1,2] and Calogero Caruso[1,2]

[1]*Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies;* [2]*Unit of Transfusion Medicine, University Hospital, University of Palermo, Italy*

Abstract: Some studies have shown that polymorphisms in the insulin growth factor-1 (IGF-1) signaling pathway genes could influence human longevity. However, the results of different studies are often inconsistent. Our aim was to investigate by systematic review and meta-analysis the association of the common polymorphisms defining the genetic variability of the IGF-1 signaling pathway associated with human longevity. Eleven studies investigating the association between the polymorphisms in the IGF-1 signaling pathway genes (IGF-1, IGF-1 receptor (IGF-1R), Forkhead box O3A (FOXO3A) and Silent mating type Information Regulation 1 (SIRT1) and longevity were found and analyzed. The modelfree approach was applied to meta-analyze these studies. No association was reported between the single nucleotide polymorphisms (SNPs) of IGF-1 and longevity in the only available study. The meta-analysis of available data from four studies, showed a significant association with the IGF-1R polymorphism rs2229765, suggesting that subjects with the Abearing genotype have a greater chance of longevity. Concerning the five studies on FOXO3A SNPs, for the rs2764264 a significant association with longevity was observed for C allele when only males were included in the analysis. Statistically significant results were obtained for other SNPs as well, *i.e.* rs2802292 (G allele), rs9400239 and rs479744 (T and A alleles, respectively). For rs9400239 the association was observed in long lived males with a lower odds ratio than in centenarians, while in rs479744 a significant association was highlighted in centenarians. Concerning SIRT1, no association between the SNPs under study and longevity was observed in the only available report. Current findings

INTRODUCTION

Genetic background represents an integral part of successful ageing and longevity, as emphasized by many studies on long-living individuals (LLI) and centenarian offspring, supporting a role for certain genes in long life. Established data are available only for Apolipoprotein E (APOE), but classic case-control studies (genome wide association studies) and high throughput sequencing identified several other genetic variants possibly associated with longevity. In recent years, ageing research has focused on the Insulin/Insulin-like growth factor-1 (IGF-1) signaling pathway, *i.e.* IGF-1, IGF-1 receptor (IGF-1R), Forkhead box O (FOXO) 3A, and Silent mating type Information Regulation 1 (SIRT1), because of its conserved evolutionary correlation with life-span extension in model organisms, such as yeast, nematodes, fruit flies and mice (Fig. 1). Many gene mutations, in particular single nucleotide polymorphisms (SNPs), associated with longevity or with increased life-span, were identified in gene encoding proteins that take part in this metabolic pathway. Moreover, the same effects on life-span were observed in different animal models, manipulating orthologue genes [1-4].

In these models, calorie restriction, causing life-span extension and IGF-1 signaling reduction, is associated with decreased circulating levels of IGF-1 [3]. In human beings, ageing is associated with lower circulating levels of IGF-1 [5], and in longevous individuals IGF-1R has been correlated with modulation of human life-span through the attenuation of IGF-1 signaling [6].

Both IGF-1 and IGF-1R polymorphisms theoretically modulating the IGF-1 pathway have been studied for their correlation with longevity, but the evidence to date is not conclusive [6, 7-10].

The IGF-1 pathway downstream Transcription Factor (TF), FOXO3A, has also been extensively studied for its role in longevity (Fig. 1). This gene belongs to the forkhead family and encodes a TF with the typical domain of this family, forkhead box, a conserved DNA-binding domain. It is one of the orthologues of *daf-16* in *C. elegans*, a TF involved in stress resistance and longevity [11, 12]. Some FOXO3A SNPs have been associated with longevity in different ethnic populations. In particular, certain variants were found in

*Address correspondence to this author at the U.O. di Medicina Trasfusionale, AOUP Paolo Giaccone, Via del Vespro 129, 90127 Palermo, Italy; Tel: +390916553222; Fax: +390916553230; E-mail: danilodibona@yahoo.it

1875-6212/14 $58.00+.0 0

suggest that both IGF-1R and FOXO3A polymorphisms could be associated with longevity. The high degree of between-study heterogeneity and the low number of available studies underline the need for further methodologically adequate analyses to confirm this evidence.

Keywords: FOXO3A, IGF-1; IGF-1R, longevity, meta-analysis, SIRT1, SNP.

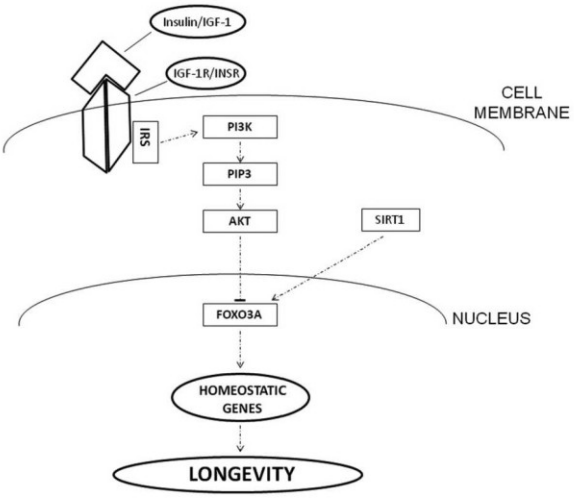


Fig. (1). Insulin/IGF1 signaling pathway and its implication in longevity. The Insulin/Insulin growth factor-1 (IGF-1) signaling pathway has a critical role in the determination of longevity. The bond of Insulin/IGF-1 to the specific receptor (IGF-1R/INSR) activates the phosphatidylinositol-3'kinase (PI3K) through the insulin related substrate (IRS). It leads to the activation of AKT, through phosphatidylinositol(3,4,5)-trisphosphate, that, in turn, inhibits Forkhead box O3A (FOXO3A), whereas Silent mating type Information Regulation 1 (SIRT1) activates it. FOXO3A acts as a transcription factor, activating the expression of many homeostatic genes. Several variants which reduce this signaling have been identified: some studies have shown their association with longevity (see text).

nonagerians and with higher frequency in centenarians, highlighting their relevant role in successful ageing. One explanation may be the increased activity of FOXO3A on downstream genes involved in survival [13-16].

In addition, FOXO3A interacts with sirtuins, a family of histone deacetylase enzymes, identified as anti-ageing molecules in model organisms (Fig. **1**). SIRT1, one of the seven human sirtuin isoforms, called SIRT1-SIRT7, deacetylates FOXO3A, modulating its response to oxidative stress [17].

On the basis of findings from experimental and animal models, some human studies sought to demonstrate an association between specific SNPs involved in modulation of Insulin/IGF and longevity. However, the sample size of most of the studies is inadequate and the results often inconsistent.

The aim of this study was to review the studies available to date on the correlation between the polymorphisms in genes involved in IGF-1 pathway and human longevity.

When possible, we used a meta-analytic approach to quantitatively synthesize the possible effect of each SNP and to reconcile the study inconsistencies.

METHODS Selection of Studies

The primary source of studies addressing the role of Insulin/IGF-1 pathway polymorphisms in longevity was the PUBMED database from January 2003 to March 2013, limited to English language literature. The medical subject headings used for PUBMED search were "IGF-1", "IGF-1R", "FOXO3A", "SIRT1", "polymorphisms", and "longevity". The specific "SNPs" of the "Insulin/IGF-1 pathway" genes, "rs2288377, rs5742612, rs35767, for IGF-1", "rs2229765, for IGF-1R", "rs2764264, rs2802292, rs1226094, rs7762395, rs9400239, rs479744, for FOXO3A", and "rs3758391, rs2273773, for SIRT1".

The abstracts found were read to identify studies examining the association between the above mentioned SNPs and longevity in healthy LLI or centenarians. We also performed a manual search of references cited in published articles. The studies were read in their entirety to assess their appropriateness for inclusion in the meta-analysis.

Any human population-based association study, regardless of sample size, was included if it met the following criteria: 1) case-control study; 2) there were at least two comparison groups of which one consisted of long-living individuals; 3)

tested association between longevity and SNPs; 4) available allelic/genomic frequencies and reference SNP ID number (rs#).

Data Extraction

Extraction of data was performed independently by GA, CV and DDB who compared results and agreed on a consensus; disagreements were settled by discussion.

Table 1. Main features of the studies analyzed

Gene	SNP	Studies	Ethnicity	Cases				Controls			
				N	F	M	Age range or mean age (±SD)	N	F	M	Age range or mean age (±SD)
IGF-1	rs2288377	Xie *et al.* '08[a]	Han	485	239	246	94.92±3.15	392	212	180	56.5±10.1
	rs5742612	Xie *et al.* '08[a]	Han	485	239	246	94.92±3.15	392	212	180	56.5±10.1
	rs35767	Xie *et al.* '08[a]	Han	485	239	246	94.92±3.15	392	212	180	56.5±10.1
IGF-1R	rs2229765	Bonafè *et al.* '03[b]	Italian	162	NA	NA	86-109	248	NA	NA	17-85
		Suh *et al.* '08[c]	Ashkenazi	79	79	0	95-108	161	161	0	79.5
		Albani *et al.* '09[d]	Italian	222	133	89	85-106	288	141	147	70-85
		Barbieri *et al.* '12[e]	Italian	183	NA	NA	96±4	488	NA	NA	49±16
FOXO3A	rs2764264	Willcox *et al.* '08[f]	Japanese	213	0	213	95-106	402	0	402	73-81
		Anselmi *et al.* '09[g]	Italian	480	199	281	90-109	335	140	195	18-48
		Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs2802292	Willcox *et al.* '08[f]	Japanese	213	0	213	95-106	402	0	402	73-81
		Anselmi *et al.* '09[g]	Italian	480	199	281	90-109	335	140	195	18-48
		Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs1226094	Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs7762395	Flachsbart *et al.* '09[i]	German	1031	764	267	95-110	731	NA	NA	60-75
		Flachsbart *et al.* '09[i]	French	535	NA	NA	103.8	553	NA	NA	18-70
		Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs9400239	Flachsbart *et al.* '09[i]	German	1031	764	267	95-110	731	NA	NA	60-75
		Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs479744	Flachsbart *et al.* '09[i]	German	1031	764	267	95-110	731	NA	NA	60-75
		Soerensen *et al.* '10[h]	Danish	1089	776	313	92-103	736	365	371	46-67
	rs1935949	Pawlikowska *et al.* '09[j]	Ashkenazi	383	286	97	95-108	363	207	156	43-94
	rs4946935	Pawlikowska *et al.* '09[j]	Ashkenazi	383	286	97	95-108	363	207	156	43-94
SIRT1	rs3758391	Flachsbart *et al.* '06[k]	German	1026	NA	NA	95-109	547	NA	NA	60-75
	rs2273773	Flachsbart *et al.* '06[j]	German	1026	NA	NA	95-109	547	NA	NA	60-75

a. [7]; b. [8]; c. [6]; d. [9]; e. [10]; f [13]; g. [16]; h. [15]; i. [14]; j. [19].; k. [20].

NA: not assigned; SNP: single nucleotide polymorphism; SD: standard deviation; IGF-1: insulin growth factor-1; IGF-1R: insulin growth factor-1 receptor; FOXO3A: forkhead box O3A; SIRT-1: Silent mating type Information Regulation Type 1.

Statistical Analysis

For meta-analysis the data were analyzed using *Review Manager,* version 5.1, a statistical software package for managing and analyzing all aspects of a Cochrane Collaboration systematic review (The Cochrane Collaboration, Oxford, UK, 1999). Controls were assumed to be as young or younger subjects compared to long-living individuals, which represented the cases. But the cut-off value between cases and controls varied greatly among individual studies. The overall odds ratio (OR) between the frequencies of alleles in both cases and controls was estimated with models based on both fixedeffects and random-effects assumptions. The fixed effects model considers only within-study variability. The random effects model uses weights that include both the within-study and between-study variance. Because of the high heterogeneity between the populations of most of the studies included in this meta-analysis, we have presented the results of random-effects models that are the most conservative ones [18]. The 95% Confidence Interval (95% CI) of the OR was also calculated.

RESULTS Characteristics of the Studies

The main features of the studies analyzed in our paper are listed in Table **1**.

IGF-1

Only one study on the association between IGF-1 rs2288377, rs5742612, rs35767 SNPs and longevity was identified by our search strategy [7]. This study was conducted on a Chinese Han population. 485 cases of LLI and 392 controls were analyzed. The mean age was 94.92±3.15 years for cases and 56.5±10.1 for controls.

IGF-1R

Four case-control studies on the association between IGF-1R rs2229765 and longevity were identified by our search strategy [6, 8-10]. Three out of four studies were conducted on an Italian population [8-10]. The remaining study was conducted on Ashkenazi Jews in North America [6]. All the analyzed studies have case-control design with a total number of 646 cases and 1185 controls. In two out of four studies the number of males and females is not reported, while in the Suh *et al.* study only females were studied [6]. The study sample size varied from 240 to 671. The age range varied from 85 to 109 years for cases and from 17 to 85 years for controls.

FOXO3A

Five case-control studies on the association between FOXO3A SNPs (rs2764264, rs2802292, rs1226094, rs7762395, rs9400239, rs479744, rs1935949 and rs4946935) and longevity were identified [13-16, 19]. The studies were conducted on different Caucasian populations, except the Willcox *et al.* study [13], which was conducted on Japanese, and the Pawlikowska *et al.* study [19], which was conducted on Ashkenazi. The Willcox *et al.* study [13] was conducted only on male subjects; the Anselmi *et al.* and Soerensen *et al.* studies [15, 16] separately analyzed data from males and females. The Pawlikowska *et al.* study [19] did not separately analyze data from males and females. The Flachsbart *et al.* study [14] did not report male/female percentage for controls. The study sample size varied from 615 to 1825. The age range varied from 90 to 110 years for cases and 18 to 94 years for controls.

SIRT1

Only one study on the association between SIRT1 rs3758391 and rs2273773 SNPs and longevity was identified by our search strategy [20]. This study was conducted on a German population. 1026 cases and 547 controls were analyzed. The study did not report male/female percentage both for cases and controls. The age range varied from 95 to 109 years for cases and from 60 to75 years for controls.

Meta-Analysis

The data concerning the association of genes involved in IGF-1 signaling pathway with longevity are reported in Table **2**.

IGF-1R

Four studies are available for the inclusion in the metaanalysis of the association between the rs2229765 SNP (3174 G>A) and longevity [6, 8-10]. The effect for the A allele and the A-bearing genotype of IGF-1R, suggests favoring longevity, and was estimated for each study. Regarding the allelic comparison (A vs. G), three out of four studies showed a favorable effect on longevity [6, 9, 10], while, the Bonafè *et al.* study [8] showed a detrimental effect on longevity [6, 8-10] (Fig. **2A**). The pooled summary OR for the allelic comparison is 1.14 (A vs. G, 95% CI: 0.82–1.59; p=0.43) with no significant statistical result using the random-effects model. In contrast, when we analyze subjects with A-bearing genotype, the summary OR is 1.73 (AA + AG vs. GG, 95% CI: 1.16–2.58; p=0.007) with a statistically significant result using the random-effects model, suggesting that subjects with low IGF-1 level associated genotypes (AA or AG) have a greater chance to achieve longevity. There is evidence of heterogeneity between the results of individual studies (A vs. G: I^2=82%; AA + AG vs. GG: I^2=68%) (Fig. **2A** & **2B**). The Bonafè *et al.* study [8] is the influential one for the allelic comparison, since removing it changes the heterogeneity from 82% to 0%, while the Suh *et al.* study [6] is the influential one for the genotypic comparison, since removing it changes the heterogeneity from 68% to 0%. The exclusion of the Suh *et al.* study [6] from the genotypic analysis does not change the overall result (OR without Suh *et al.*: 1.43 95% CI 1.14-1.79), the exclusion of the Bonafè *et al.* study [8] from the allelic comparison changes the overall result (OR without Bonafè *et al.*: 1.35 95% CI 1.15-1.58), with a statistically significant result as well.

A statistically non-significant result for the allelic comparison (A vs. G: OR: 1.10, 95% CI: 0.72–1.67; p=0.65) but a statistically significant effect for the genotypic comparison (AA + AG vs. GG: OR: 1.43, 95% CI: 1.43–

1.79; p=0.002) is obtained when the analysis is limited to the Italian population [8-10].

FOXO3A

Three studies are available for the inclusion in the metaanalysis of the association between the rs2764264 and rs2802292 SNPs and longevity [13, 15, 16]. Data are only suitable for allelic comparison. For the rs2764264 we report a statistically non-significant effect for the C allele, putatively favoring longevity (OR [C]: 1.20, 95% CI: 0.95–1.51; p=0.12, I^2 52%) (Fig. **3A**), but a statistically significant result when only males are included in the analysis (OR [C]: 1.38, 95% CI: 1.13–1.69; p=0.002; I^2 0%), showing that the C allele is associated with longevity only in males (Fig. **3B**).

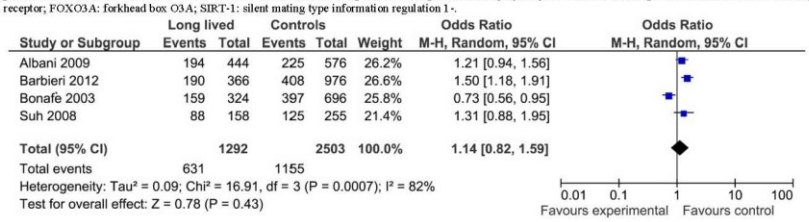
For the rs2802292 SNP, we report statistically significant results for an association between the G allele and longevity, when we compare both the overall population and the male population (overall population OR [G]: 1.37, 95% CI: 1.03–1.83; p=0.03, I^2 52%; male population OR [G]: 1.49, 95% CI: 1.22–1.82; p=0.0001, I^2 0%). But for this SNP, two out of three studies report data only for males [13, 16]. Data for the rs7762395, rs9400239 and rs479744 are reported in two studies, the Soerensen *et al.* and Flachsbart *et al.* studies [14, 15]. For the rs7762395, we do not report a statistically significant association between the A allele and longevity both for LLI (OR [A]: 1.15, 95% CI: 0.96–1.38; p=0.14; I^2 0%), and centenarians (OR [A]: 1.17, 95% CI:

Table 2. Association between polymorphisms of genes involved in the IGF (insulin growth factor) signaling pathway to longevity

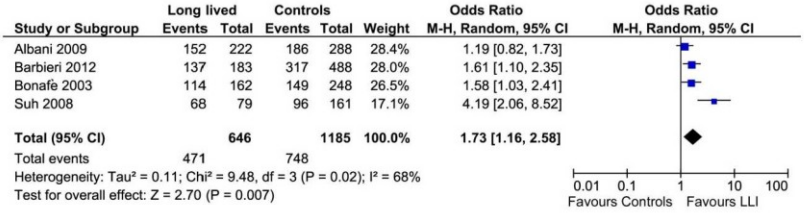
Gene	SNP	Studies	Nucleotide change	Cases/ controls	Summary Results	Association (Pos/Neg)	Findings
IGF-1	rs2288377	Xie *et al.* '08[a]	T>A	485/392	-		No association
	rs5742612	Xie *et al.* '08[a]	C>T	485/392	-		No association
	rs35767	Xie *et al.* '08[a]	T>C	485/392	-		No association
IGF-1R	rs2229765	Bonafè *et al.* '03[b]	A>G	646/1185	OR = 1.73 (95%CI 1.16, 2.58.)	4/0	The presence of at least an A allele favors longevity
		Suh *et al.* '08[c]					
		Albani *et al.* '09[d]					
		Barbieri *et al.* '12[e]					
FOXO3A	rs2764264	Willcox *et al.* '08[f] Anselmi *et al.* '09[g] Soerensen *et al.* '10[h]	C>T	1782/1473 807/968	OR (All) = 1.20 (95%CI 0.95, 1.51) OR (Males) = 1.38 (95%CI 1.13, 1.69)	3/0 3/0	The C allele is associated to longevity in males
	rs2802292	Willcox *et al.* '08[f] Anselmi *et al.* '09[g] Soerensen *et al.* '10[h]	G>T	1782/1473 807/968	OR (All) = 1.37 (95%CI 1.03, 1.83) OR (Males) = 1.49 (95%CI 1.22, 1.82)	3/0 3/0	The G allele is associated to longevity. Data available only for males in 2 out of 3 studies
	rs1226094	Soerensen *et al.* '10[h]	C>T	1089/736 313/371	OR (All) = 1.13 (95%CI 0.92, 1.40) OR (Males) = 1.38 (95%CI 1.08, 1.75)	1/0	The T allele is associated to longevity in males
	rs7762395 - LLI	Flachsbart *et al.* (GER) '09[i] Soerensen *et al.* '10[h]	G>A	2120/1467	OR (All) = 1.15 (95%CI 0.96, 1.38)	2/0	No significant association to longevity for LLI
	-Centenar.	Flachsbart *et al.* (FRA) '09[i] Flachsbart *et al.* (GER) 09[i] Soerensen *et al.* '10[h]	G>A	1066/2020	OR (All) = 1.17 (95%CI 0.95, 1.43)	3/0	No significant association

	rs9400239 -LLI	Flachsbart *et al.* (GER) '09[i]	T>C	2120/1467	OR (All) = 1.13 (95%CI 0.98, 1.32)	2/0	Significant association only for males
				1344/1102	OR (Males)* = 1.20 (95%CI 1.01, 1.43)	2/0	
	-Centenar.	Soerensen *et al.* '10[h]	T>C				
				531/1467	OR (All) = 1.32 (95%CI 1.06, 1.64)	2/0	
		Flachsbart *et al.* (GER) 09[i]		418/1102	OR (Males)* = 1.37 (95%CI 1.07, 1.76)	2/0	Significant association in centenarians
		Soerensen *et al.* '10[h]					
	rs479744 -LLI	Flachsbart *et al.* (GER) '09[i] Soerensen *et al.* '10[h]	A>C	2120/1467	OR (All) = 1.16 (95%CI 0.99, 1.37)	2/0	No significant association
				1344/1102	OR (Males)* = 1.22 (95%CI 1.00, 1.48)	2/0	
	-Centenar.		A>C				
		Flachsbart *et al.* (GER) '09[i]		531/1467	OR (All) = 1.41 (95%CI 1.11, 1.79)	2/0	Significant association in centenarians
		Soerensen *et al.* '10[h]		418/1102	OR (Males)*= 1.41 (95%CI 1.07, 1.86)	2/0	
	rs1935949 - Centenar.	Pawlikowska *et al.* '09[j]	NA	383/363	OR (All) = 1.36 (95%CI 1.05-1.74)		Significant association in centenarians
	rs4946935 -Centenar	Pawlikowska *et al.* '09[j]	NA	383/363	OR (All) = 1.33 (95%CI 1.03-1.72)		Significant association in centenarians
SIRT1	rs3758391	Flachsbart *et al.* '06[k]	T>C	1026/547	-		No association
	rs2273773	Flachsbart *et al.* '06[k]	T>C	1026/547	-		No association

Results LLI and controls are reported for all the comparisons. All the comparisons are allelic based, except for IGF-1R, which was a genotypic comparison. For the Flachsbart *et al.* 2009 study data on centenarians, available separately for French and German populations, are also reported. The minor allele is underlined.
*For this comparison the OR (males) data from males were available only from the Sorensen's *et al.* study. The Flachsbart *et al.* (GER) study included data from both males and females. a. [7]; b. [8]; c. [6]; d. [9]; e. [10]; f [13]; g. [16]; h. [15]; i. [14]; j. [19]; k [20].
LLI: long-lived individuals; OR: odds ratio; CI: confidence interval; NA: not assigned; SNP: single nucleotide polymorphism; IGF-1: insulin growth factor-1; IGF-1R: insulin growth factor-1 receptor; FOXO3A: forkhead box O3A; SIRT-1: silent mating type information regulation 1 -.

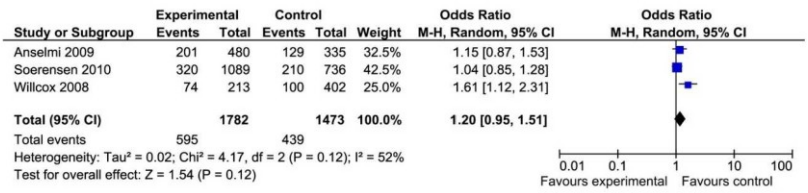


A

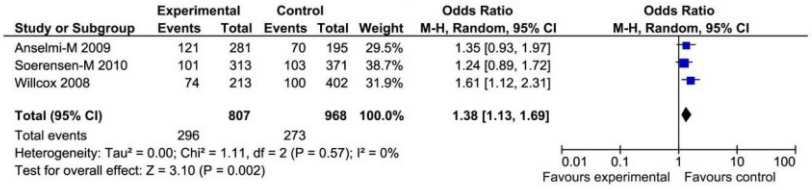


B

Fig. (2). Meta-analysis of four case-control studies of the IGF-1R rs2229765 polymorphism and longevity using the random-effects model. The odds ratio and 95% confidence interval (CI) for the effect of A vs. G allele (**2A**) and the AA + AG vs. GG genotypes (**2B**) on longevity are plotted on the two graphs. Studies are arranged chronologically based on the year of publication. M-H: Manten-Hanzel; C.I.: Confidence interval.



A



B

Fig. (3). Meta-analysis of three case-control studies of the FOXO3A rs2764264 polymorphism and longevity using the random-effects model. The odds ratio and 95% confidence interval (CI) for the effect of the C allele on longevity for the whole population (**3A**) and for males only (**3B**) are plotted on the two graphs. Studies are arranged chronologically based on the year of publication. M-H: Manten-Hanzel; C-I: Confidence Interval.

0.95–1.43; p=0. 13, $I^2$0%). For the rs9400239 and rs479744, we show a statistically significant association between the minor allele (T for rs9400239 and A for rs479744) and longevity when only male LLI from the Soerensen *et al.* study are included (rs9400239 OR [T]: 1.20, 95% CI: 1.01–1.43; p=0.04; rs479744 OR [A]: 1.22, 95% CI: 1.00–1.48; p=0.05), but not for the entire population (rs9400239 OR [T]: 1.13, 95% CI: 0.98–1.32; p=0.10; rs479744 OR [A]: 1.16, 95% CI: 0.99–1.37; p=0.07).

For the last two SNPs (rs9400239 and rs479744) we have also calculated the effect of the minor allele in a centenarian population showing a statistically significant association not only in males (rs9400239 OR [T]: 1.37, 95% CI: 1.07–1.76; p=0.01; rs479744 OR [A]: 1.41, 95% CI: 1.07–1.86; p=0.01), but for the entire population (rs9400239 OR [T]: 1.32, 95% CI: 1.06–1.64; p=0.01; rs479744 OR [A]: 1.41, 95% CI: 1.11–1.79; p=0.005).

Single Study Results (Table 2)

IGF-1. No association was reported between the rs2288377, rs5742612, and rs35767 SNPs of the IGF-1 gene and longevity in the Xie *et al.* study [7].

FOXO3A. For the FOXO3A rs1226094 SNP, a statistically significant association between the T allele and longevity was reported in males in the Soerensen *et al.* study [15]. For the FOXO3A rs1935949 and rs4946935 SNPs, a statistically significant association with centenarians was reported in the Pawlikowska *et al.* study [19].

SIRT-1. No association was reported between the rs3758391 and the rs2273773 of the SIRT-1 gene and longevity in the Flachsbart *et al.* study [20].

DISCUSSION

Ageing is considered the product of an interaction among genetic, epigenetic, stochastic, lifestyle and environmental factors which in turn influence longevity, *i.e.* the ability to survive beyond the species-specific average age of death [21-23]. A variety of models in lower organisms and in mammals demonstrate that single genetic mutations are able to increase life-span. In particular, mutations in genes that are homologous to those encoding proteins involved in mammalian Insulin/IGF-1 pathway affect life-span in yeast, nematodes and fruit flys [3, 24]. Fig. (**1**) shows the principal components of this pathway. In animal models all the effects of this pathway on the extension of life-span depend on its

decreased activity leading to a reduced phosphorylation of *daf-16*/FOXO TFs that increases translocation to the nucleus and their activity [3, 24]. During evolution, the pathway has diverged from a single receptor in invertebrates to multiple receptors and more complicated pathways and regulatory networks in mammals. However, a series of genetic manipulations in mice has provided evidence that this pathway also affects ageing and longevity in mammals [3]. The effect of FOXO on life-span may be linked to its action as a transcription factor on multiple homeostatic pathways in response to decreased Insulin/IGF-1 signaling [3].

Interestingly, other genes that increase life-span, *i.e.* the enzymes histone deacetylase sirtuins, when overexpressed, interact with FOXO. In particular, SIRT1 deacetylates FOXO3A and modulates its response to oxidative stress [2, 25].

In humans, several case-control studies have been performed to establish an association between longevity and genetic polymorphisms in this pathway including sirtuins.

There is substantial but not conclusive evidence of an effect in some genes of this pathway on the achievement of longevity. So in the present paper we have performed a systematic review and meta-analysis with the aim of reconciling the study inconsistencies.

Eleven studies investigating the association between the SNPs in the Insulin/IGF-1 signaling pathway genes, *i.e.* IGF1, IGF-1R, FOXO3A and SIRT1 and longevity were found and analyzed [6-10, 13-16, 19, 20] (Table **1**). The model-free approach was applied for the meta-analysis (Table **2**, Figs. **2, 3A & B**).

No association was reported between the SNPs of IGF-1 and longevity in the available studies although these SNPs could affect IGF-1 serum levels, known to modulate ageing and longevity [7]. On the other hand, higher circulating levels of IGF-1 have also been associated with longer leukocyte telomere length, a key biomarker of human ageing, in healthy subjects [26].

In contrast, the available data from four studies [6, 8-10], show a statistically significant association of longevity with the IGF-1R polymorphism rs2229765, suggesting that subjects with the A-bearing genotype responsible for reduced signal transduction have a higher chance of achieving longevity. The relevance of IGF-1R for longevity is further suggested by a meta-analysis performed on participants from the Study of Osteoporotic Fractures and Cardiovascular Health Study that shows a significant association with longevity for the rs2272037 SNP [19].

Concerning the five studies on FOXO3A SNPs [13-16, 19], for the rs2764264 a significant association with longevity is observed for the C allele when only males are included in the analysis. The same is true for other SNPs, *i.e.* rs2802292, G allele (but for this allele 2 out of 3 studies report data only for males) and for the rs9400239 and rs479744 (T and A alleles, respectively) that were reported only in 2 studies. This result is not surprising because it has been claimed that males and females follow different strategies to attain longevity and several case-control studies have been positive only in males [4, 27]. Concerning the C allele it is interesting to note that in the Willcox *et al.* study, the C-carrier cases were healthier at the baseline examination despite the fact that they were, on average, 11 years older [13].

The rs479744 (A allele) and the rs9400239 (T allele) SNPs have been significantly associated with longevity in centenarian populations, as well as the rs1935949 and rs4946935 SNPs. These results are not surprising, because centenarians represent the survival tail of the population [21]. Thus, they may be particularly enriched for beneficial variants in longevity assurance genes. In addition, it is relevant that the population based Leiden 85-plus study also found a FOXO3A haplotype, but no single SNP associated with increased mortality [28].

Concerning SIRT1, no association between the SNPs under study and longevity was observed in the only available report [20].

On the whole, data obtained by our study clearly demonstrate that two players of Insulin/IGF-1 pathway are associated with human longevity, *i.e.* IGF-1R and FOXO3A. Since drugs able to modulate this pathway are under scrutiny, these data suggest the possibility that successful ageing could be pharmacologically modulated. However, we have no clue as to how these SNPs could possibly act.

In any case, extensive studies of the whole pathway are needed, including the recently reported gene codifying calcium/calmodulin-dependent protein kinase IV (CAMKIV). It has been claimed that a variant of this gene is associated with longevity by influencing CAMKIV protein expression, hence allowing the activation of FOXO3A by the native protein [23].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This meta-analysis was entirely supported by the authors' respective institutions. G.A. and C.V. are PhD students (Tutor GC, Supervisor CC), and this paper is submitted in partial fulfillment of their PhD degree.

REFERENCES

[1] Beekman M, Blanché H, Perola M, *et al.* Genome-wide linkage analysis for human longevity: Genetics of Healthy Aging Study. Aging Cell 2013; 12: 184-93.

[2] Franceschi C, Olivieri F, Marchegiani F, *et al.* Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. Mech Ageing Dev 2005; 126: 351-61.

[3] Ziv E, Hu D. Genetic variation in insulin/IGF-1 signaling pathways and longevity. Ageing Res Rev 2011; 10: 201-4.

[4] Candore G, Caruso C, Colonna-Romano G. Inflammation, genetic background and longevity. Biogerontology 2010; 11: 565-73.

[5] Bartke A. Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. Endocrinology 2005; 146: 3718-23.

[6] Suh Y, Atzmon G, Cho MO, *et al.* Functionally significant insulinlike growth factor I receptor mutations in centenarians. Proc Natl Acad Sci USA 2008; 105: 3438-42.

[7] Xie L, Gong YY, Lian SG, *et al.* Absence of association between SNPs in the promoter region of the insulin-like growth factor 1 (IGF-1) gene and longevity in the Han Chinese population. Exp Gerontol 2008; 43: 962-5.

[8] Bonafè M, Barbieri M, Marchegiani F, *et al.* Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span control. J Clin Endocrinol Metab 2003; 88: 3299-304.

[9] Albani D, Batelli S, Polito L, *et al.* A polymorphic variant of the insulin-like growth factor 1 (IGF-1) receptor correlates with male longevity in the Italian population: a genetic study and evaluation of circulating IGF-1 from the "Treviso Longeva (TRELONG)" study. BMC Geriatr 2009; 21; 9: 19.

[10] Barbieri M, Boccardi V, Esposito A, *et al.* A/ASP/VAL allele combination of IGF1R, IRS2, and UCP2 genes is associated with better metabolic profile, preserved energy expenditure parameters, and low mortality rate in longevity. Age 2012; 34: 235-45.

[11] Gems D, McElwee JJ. Aging: Microarraying mortality. Nature 2003; 424: 259-61.

[12] Kenyon C. The plasticity of aging: Insights from long-lived mutants. Cell 2005; 120: 449-60.

[13] Willcox BJ, Donlon TA, He Q, *et al.* FOXO3A genotype is strongly associated with human longevity. Proc Natl Acad Sci USA 2008; 105: 13987-92.

[14] Flachsbart F, Caliebe A, Kleindorp R, *et al.* Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci USA 2009; 106: 2700-5.

[15] Soerensen M, Dato S, Christensen K, *et al.* Replication of an association of variation in the FOXO3A gene with human longevity using both case-control and longitudinal data. Aging Cell 2010; 9: 1010-7.

[16] Anselmi CV, Malovini A, Roncarati R, *et al.* Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. Rejuvenation Res 2009; 12: 95-104.

[17] Brunet A. The multiple roles of FOXO transcription factors. Med Sci (Paris) 2004; 20: 856.

[18] DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986; 7: 177–188.

[19] Pawlikowska L, Hu D, Huntsman S, *et al.* Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. Aging Cell 2009; 8: 460-72.

[20] Flachsbart F, Croucher PJ, Nikolaus S, *et al.* Sirtuin 1 (SIRT1) sequence variation is not associated with exceptional human longevity. Exp Gerontol 2006; 41: 98-102.

[21] Caruso C, Passarino G, Puca A, Scapagnini G. "Positive biology": the centenarian lesson. Immun Ageing 2012; 9: 5.

[22] Montesanto A, Dato S, Bellizzi D, Rose G, Passarino G. Epidemiological, genetic and epigenetic aspects of the research on healthy ageing and longevity. Immun Ageing 2012; 9: 6.

[23] Malovini A, Illario M, Iaccarino G, Villa F, Ferrario A, Roncarati R. Association study on long-living individuals from Southern Italy identifies rs10491334 in the CAMKIV gene that regulates survival proteins. Rejuvenation Res 2011; 14: 283-91.

[24] Pan Z, Chang C. Gender and the regulation of longevity: implications for autoimmunity. Autoimmun Rev 2012; 11: A393-403.

[25] Guarente L. Franklin H. Epstein Lecture: Sirtuins, aging, and medicine. N Engl J Med 2011; 364: 2235-44.

[26] Barbieri M, Paolisso G, Kimura M, *et al.* Higher circulating levels of IGF-1 are associated with longer leukocyte telomere length in healthy subjects. Mech Ageing Dev 2009; 130: 771-6.

[27] Capri M, Salvioli S, Monti D, *et al.* Human longevity within an evolutionary perspective: the peculiar paradigm of a postreproductive genetics. Exp Gerontol 2008; 43: 53-60.

[28] Kuningas M, Mägi R, Westendorp RG, Slagboom PE, Remm M, Van Heemst D. Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. Eur J Hum Genet 2007; 15: 294-301.

Received: August 1, 2013 **Revised:** October 21, 2013 **Accepted:** October 31, 2013

1.5 ASSOCIATION OF KLOTHO POLYMORPHISMS WITH HEALTHYAGEING: A SYSTEMATIC REVIEW and META-ANALYSIS

REJUVENATION RESEARCH
Volume 17, Number 2, 2014 [a]
Mary Ann Liebert, Inc.
DOI: 10.1089/rej.2013.1523

Association of Klotho Polymorphisms with Healthy Aging: A Systematic Review and Meta-Analysis

Danilo Di Bona,[1,2] Giulia Accardi,[1] Claudia Virruso,[1] Giuseppina Candore,[1,2] and Calogero Caruso[1,2]

Abstract

Today it is clearly evident that genetic background constitutes an integral part of aging and longevity. Many studies on long-lived people have been conducted emphasizing the role of certain genes in long life. Classic case–control studies, genome-wide association studies, and high-throughput sequencing have permitted identification of a variety of genetic variants seemingly associated with longevity. Over the years, aging research has focused on the insulin/insulin-like growth factor-1 (IGF-1) signaling pathway because of its evolutionarily conserved correlation with life-span extension in model animals. Indeed, many single-nucleotide polymorphisms (SNPs) associated with longevity were identified in genes encoding proteins that take part in this metabolic pathway. Closely related to this pathway is the Klotho gene. It encodes a type-I membrane protein expressed in two forms, membrane and secreted. The latter form suppresses oxidative stress and growth factor signaling and regulates ion channels and transporters. In particular, its over-expression seems to be able to suppress insulin/IGF-1 signaling extending life span. Thus, our aim was to assemble the results in the literature concerning the association between the functional variant of the Klotho "KL-VS" stretch, which contains six polymorphisms in linkage disequilibrium, and successful aging to quantify the possible effect of the variants. The results of our systematic review indicate that the Klotho KL-VS variant is associated with healthy aging.

Introduction

Aging and longevity are multi-factorial events. Genetic, epigenetic, stochastic, and environmental factors seem to have a crucial role in aging and longevity. Approximately 25% of the overall variation in human life span can be attributed to genetic factors, which become more relevant for extreme longevity. Conditioning factors, which arise in the first part of life, account for another 25% of such variability; life circumstances at adult and old age may account for about the remaining 50%. Concerning the role of genetics, three approaches—candidate gene approach, genome-wide association studies (GWAS), and meta-analysis— have been used to assess the contribution of different polymorphisms.[1–3]

The candidate gene approach is a hypothesis-driven method widely employed by case–control studies. The genotype and allele frequencies of two populations are compared—one affected and one unaffected by a complex trait, like longevity. If the identified allelic variants are more prevalent in the population in study as compared to controls, these genotypes are associated with the complex trait.[4] The number of reported studies on the association between one "old" and recent data, see refs. 2 and 5). Consistent replicanetic, greatly increasing, even though a large number of these studies show inconsistent results (for an extensive analysis of different populations has been argued as strong evidence of a true association. However, the genetics of aging and longevity is complex and may alter according to gender and country. The lack of replication may not necessarily imply a false association, but might simply point to the need for more studies in certain populations or more detailed study of the function of a gene, taking into account different gene environment interactions, since, as previously stated, aging and longevity phenotypes are strongly affected by lifestyle and environmental factors and by complex epistatic and pleiotropic effects in several genes.[1,6,7]

GWAS consist of scanning whole-genome analysis markers to find variants associated with the trait of interest using a case–control study. It is important to note that the finding of common genetic variants with low allelic frequency across studies is consistently difficult because of the multitude of data that must be analyzed. In addition, population admixture may produce possible false positives

or multiple singlenucleotide polymorphisms (SNPs) and aging and longevity is due to different genetic backgrounds among ethnic groups. However, GWAS are a useful tool for the identification of

[1] [2] Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, and Unit of Transfusion Medicine, University Hospital, University of Palermo, Palermo, Italy.

complex trait-associated alleles with frequencies above 5%. Each, taken singularly, has a moderate or null effect, but it is possible to speculate that more alleles have, en bloc, a synergic rather than an additional effect.[4] By using a GWAS approach for long-living individuals, several loci have reached a genome-wide level of significance that is not always confirmed in different studies.[3,8]

However, some interesting data have been obtained recently, such as those derived by meta-analysis.[9] Metaanalysis provides a mean to quantitatively synthesize association data across studies of the same genetic variant. Thus, the use of meta-analyses has recently become an important part of genetic research, mainly to reconcile previously conducted studies that have given inconsistent results.[4,9]

The gene Klotho, aptly named for Clotho, one of the Greek Fates goddesses believed by the ancients to spin the thread of life, encodes a type-I membrane protein expressed in two forms, membrane and secreted. It was discovered about 15 years ago as a gene that, if knocked out in mice, precipitates their accelerated aging, including short life span; its over-expression suppresses aging and extends life span.[10,11] On this basis, some human studies sought to demonstrate an association between the functional variant of the Klotho ''KL-VS'' stretch that contains six polymorphisms in linkage disequilibrium, involved in modulation of its activity by influencing trafficking and catalytic activity of its secreted form and aging and longevity. However, the results have been inconsistent.[12–15]

The aim of this study was to review the studies available to date on the correlation between the KL-VS variant of the Klotho gene and human aging and longevity. We used a meta-analytic approach to quantitatively synthesize the possible effect of the variant and to reconcile the study inconsistencies.

Methods

Selection of studies

The primary source of the studies addressing the role of Klotho KL-VS variant in longevity was the PubMed database (from January, 2003, to September, 2013) limited to English language literature. The medical subject headings used for PubMed search were ''Klotho,'' ''KL-VS variant,'' ''aging,'' and ''longevity.''

The abstracts found were read to identify studies examining the association between the above-mentioned allele and aging and longevity in healthy aged subjects or centenarians. We also performed a manual search of references cited in published articles. The studies were read in their entirety to assess their appropriateness for inclusion in the meta-analysis.

Any human population-based association study, independently on sample size, was included if it met the following criteria: (1) It was a case–control study. (2) There were at least two comparison groups, of which one consisted of healthy aged or long-living individuals. (3) The study tested the association between aging and longevity and the variant.

Data extraction

Extraction of data was independently performed by D.D.B., G.A., and C.V., who compared results and agreed on a consensus. Disagreements were settled by discussion.

Statistical analysis

For meta-analysis, data were analyzed using Review Manager, version 5.1, a statistical software package for managing and analyzing all aspects of a Cochrane Collaboration systematic review (The Cochrane Collaboration, Oxford, UK, 1999). Controls were assumed to be as young or younger subjects compared to aged and long-living individuals, which represented the cases. But the cutoff value between cases and controls varied greatly among individual studies. The overall odds ratio (OR) between the frequencies of alleles in both cases and controls

was estimated with models based on both fixed-effects and random-effects assumptions. The fixed-effects model considers only withinstudy variability. The random-effects model uses weights that incorporate both the within-study and between-study variances. Because of the high heterogeneity between the populations of most of the studies included in this metaanalysis, we have presented the results of random-effects models that are the most conservative ones.[16] The 95% confidence interval (95% CI) of the OR was also calculated.

Results

Characteristics of the studies

Four studies on the association between the Klotho KL-VS variant and aging were identified by our search strategy.[12–15] The Arking et al. study[12] was performed on three different populations (Bohemian Czech, Baltimore Caucasian, Baltimore African American), thus it was considered as three different studies for the inclusion in the meta-analysis. The other three studies were performed on US Caucasians,[13] Italians,[14] and Indians.[15] The studies were conducted on 2913 aged people (from 199 to 723) and 2206 controls (from 226 to 463) on aggregate. A remarkable heterogeneity is shown in the age of enrolled populations both in cases (range, from 41 to 109 years) and controls (range, from newborn to 65 years). In regard to the Majumdar et al. study,[15] we included only the healthy control population in the meta-analysis. Thus, people 40 years old or less were considered as the control population, whereas people over 40 were considered as aged (Table 1). The frequency of the Klotho genotypes in both cases and controls reported in each of the studies included in the meta-analysis is shown in Table 2. It should be noted that in the Invidia et al. study,[14] a single SNP, 115G/A, was used to tag the KL-VS haplotype because all six SNPs occur in perfect linkage disequilibrium.

Meta-analysis

The effect for the KL-VS variant on aging, suggested as favoring longevity through a putative increase of secreted Khloto levels, was estimated for each study. Five out of six studies[12,14,15] show a little favorable effect on longevity, but only two of these reach statistical significance.[12,14] In contrast, the Novelli et al. study[13] shows a little detrimental effect on longevity. The pooled summary OR for the genotypic comparison between the wild type (wt/wt) versus the heterozygous (wt/KL-VS) variant is 1.14 (OR=1.14, 95% CI 1.00–1.30) with a marginal statistical significance (p=0.05) using the random-effects model (Fig. 1A). In contrast, when we compare the Klotho wt homozygous subjects (wt/wt) versus KL-VS-bearing genotypes (wt/KL-VS+KLVS/KL-VS), the summary OR is 1.10 (95% CI 0.97–1.25; p=0.13), suggesting that KL-VS homozygous subjects not only do not show any advantage in aging, but seem to have a disadvantage (Fig. 1B), although this comparison does not reach statistical significance. This observation seems to be confirmed by comparing KL-VS homozygous people (KLVS/KL-VS) versus Klotho wt homozygous+heterozygous subjects (KL-VS/wt+wt/wt) (OR 0.73; 95% CI 0.41–1.30; p=0.29), but, also in this case, with a result that is not statistically significant (Fig. 1C). Notably, the I^2 value for heterogeneity in this last comparison is 50%, showing a significant between-study heterogeneity compared to the first two comparisons, which do not show any between-study heterogeneity (I^2=0%).

Discussion

Our meta-analysis summarizes the evidence to date regarding the association between the Klotho KL-VS variant and aging and longevity, representing a pooled total of 2913 cases and 2206 controls. The results indicate a significant association of the variant with healthy aging and longevity, despite the serious limitations of the study.

The results of this study are subjected to many limitations, which could partially mask the true genetic effect. First of all, it must be emphasized that there is a remarkable heterogeneity between the populations included in the different studies, both in the cases and the controls. In fact, whereas the Arking et al. study[12] uses the newborn as control group, the other three studies use people under 35,[13] under 40,[15] or between 19 and 65 years.[14] There also are many differences between the aging populations, leading, in some cases, to an overlapping between cases and controls of different studies (Table 1).

The populations included in the analysis are from different ethnicities. However, a subgroup analysis exploring the effect on the Klotho genetic variant on populations of the same ethnic groups cannot be performed, given the low number of available studies. It should be noted, then, that the Arking et al. study[12] shows a genetic effect only in the Bohemian Czech population, suggesting that genetic or environmental factors could influence the observed effect. However, homozygous elderly individuals were underrepresented in the three populations under study (see below).

Although the presence of the KL-VS variant in heterozygosis is associated with aging and longevity compared to the Klotho wild-type gene, the KL-VS variant

Table 1. Clinical Characteristics of the Populations Included in the Meta-Analysis

		Cases		*Controls*	
Study (year of publication)	*Population*	n	*Age*	n	*Age*
Arking et al. 2002[12]	Bohemian Czech	415	‡75	390	Newborn
Arking et al. 2002[12]	Baltimore Caucasian	723	‡65	420	Newborn
Arking et al. 2002[12]	Baltimore African American	242	‡65	226	Newborn
Novelli et al. 2008[13]	US Caucasian	708	93–105	332	<35
Invidia et al. 2010[14]	Italian	626	66–109	463	19–65
Majumdar et al. 2010[15]	Indian	199	>40	375	<40

in homozygosis shows an opposite effect. This could be due to a true genetic effect only in heterozygous people, with a mechanism not related to the gene dose. Alternatively, it could be due to the little sample size of the KL-VS homozygous group (Fig. 1C) that hampers the reliability of the statistical analysis. This latter hypothesis is suggested by the high between-study heterogeneity and the high within-study variance observed in the comparison between KL-VS homozygous group and the KL-VS heterozygous group+wild type (Fig. 1C), with studies showing conflicting results,

Table 2. Klotho Genotypes in Case and Control Populations

Study (year of publication)	*Population*	*wt/wt*	*wt/KL-VS*	*KL-VS/KL-VS*	n
Arking et al. 2002[12] Cases	Bohemian Czech	308	103	4	415
Controls		307	73	10	390
Cases	Baltimore Caucasian	530	185	8	723
Controls		309	100	11	420
Cases	Baltimore African American	68	5	242	
Controls		156	58	12	226
Novelli et al. 2008[13] Cases	US Caucasian	517	170	21	708
Controls		241	85	6	332
Invidia et al. 2010[14] Cases	Italian	439	174	13	626
Controls		348	103	12	463
Majumdar et al. 2010[15] Cases	Indian	140	53	6	199
Controls		270	99	6	375

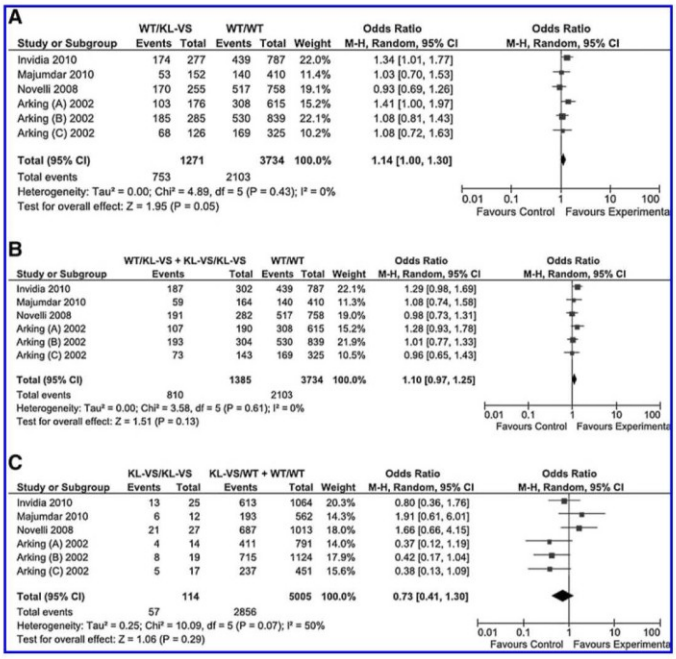


FIG. 1. (A) Meta-analysis of six case–control studies of the Klotho KL-VS polymorphism and aging using the random effects model. The odds ratio (OR) and 95% confidence interval (CI) for the effect of the wt/KL-VS versus wt/wt genotypes on aging are plotted on the graph. Studies are arranged chronologically based on the year of publication. Arking A 2002,[12] Bohemian Czech population; Arking B, Baltimore Caucasian population; Arking C, Baltimore African American population. (B) Meta-analysis of six case–control studies of the Klotho KL-VS polymorphism and aging using the random effects model. The OR and 95% CI for the effect of the KL-VS/KL-VS+ wt/KL-VS versus wt/wt genotypes on aging are plotted on the graph. Studies are arranged chronologically based on the year of publication. Arking A 2002, Bohemian Czech population; Arking B, Baltimore Caucasian population; Arking C, Baltimore African American population. (C) Meta-analysis of six case–control studies of the Klotho KL-VS polymorphism and aging using the random-effects model. The OR and 95% CI for the effect of the KL-VS/KL-VS versus wt/KL-VS+ wt/wt genotypes on aging are plotted on the graph. Studies are arranged chronologically based on the year of publication. Arking A 2002, Bohemian Czech population; Arking B, Baltimore Caucasian population; Arking C, Baltimore African American population.

different from the other comparisons (Fig. 1A, B). However, cross-sectional and prospective studies confirm a genetic model in which the KL-VS allele confers a heterozygous advantage in conjunction with a marked homozygous disadvantage with low levels of high-density lipoprotein cholesterol, high systolic blood pressure, increased risk of stroke, and early-onset coronary artery disease, and mortality.[17,18]

Finally, in the Invidia et al. study,[14] it has been demonstrated that the KL-VS variant has been observed to be increased in the elderly, but not in the group of long-living people, suggesting that this KL-VS heterozygous genotype is favorable for survival in old people; its beneficial effect decreases thereafter, becoming no more evident at the extreme ages.

Concerning the function of Klotho leading to such effects on healthy aging and longevity, the soluble form of Klotho, released from cell membranes into the serum, has homology to the family 1 glycosidases that cleave glycosidic bonds in sugars, glycolipids, and glycoproteins. It acts on several targets, including the receptor of insulin/insulin-like growth factor (IGF-1) signaling pathway.[10,11] It is noteworthy that its activity seems inversely correlated with the activity of this pathway: the decreased life span of Klotho knockout mice is rescued by inactivation of the insulin/IGF-1 pathway.[19] Thus, it is possible that secreted Klotho protein may modify glycans of the insulin/IGF-1 receptors, inhibiting their activity and/or altering their cell-surface abundance.[10,11]

The inhibition of this pathway has a critical role in the determination of longevity, and several variants that reduce

this signaling have been identified; some studies have shown their association with longevity.[9] The bond of insulin/IGF-1 to the specific receptor activates phosphatidylinositol 3kinase through the insulin-related substrate. It leads to the activation of AKT that, in turn, inhibits Forkhead box O3A (FOXO3A). FOXO3A acts as transcription factor, activating the expression of many homeostatic genes, including antioxidant catalase and mitochondrial manganese-superoxide dismutase[11,20]; hence, the inhibition of this pathway induces resistance to oxidative stress.

We can conclude that the KL-VS variant, which influences the trafficking and catalytic activity of the secreted protein, favors healthy aging and longevity by inhibiting the insulin/IGF-1 signaling pathway. In fact, adequate suppression of this pathway is an evolutionarily conserved mechanism for antiaging and life span extension because this pathway negatively regulates transcription factors FOXO involved in up-regulation of homeostatic genes.[9–11,20]

Acknowledgments

This meta-analysis has been entirely supported by the authors' respective institutions. G.A. and C.V. are PhD students (Tutor G.C., Supervisor C.C.), and this article is submitted in partial fulfillment of their PhD degree.

Author Disclosure Statement

The authors have no conflict of interest.

References

1. Caruso C, Passarino G, Puca A, Scapagnini G. ''Positivebiology'': The centenarian lesson. Immun Ageing 2012;9:5.
2. Montesanto A, Dato S, Bellizzi D, Rose G, Passarino G. Epidemiological, genetic and epigenetic aspects of the research on healthy ageing and longevity. Immun Ageing 2012; 9:6.
3. Ferrario A, Villa F, Malovini A, Araniti F, Puca AA. Theapplication of genetics approaches to the study of exceptional longevity in humans: Potential and limitations. Immun Ageing 2012;9:7.
4. Incalcaterra E, Accardi G, Balistreri CR, Caimi G, CandoreG, Caruso M, Caruso C. Pro-inflammatory genetic markers of atherosclerosis. Curr Atheroscler Rep 2013;15:329–337.
5. Salvioli S, Olivieri F, Marchegiani F, Cardelli M, Santoro A,Bellavista E, Mishto M, Invidia L, Capri M, Valensin S, Sevini F, Cevenini E, Celani L, Lescai F, Gonos E, Caruso C, Paolisso G, De Benedictis G, Monti D, Franceschi C. Genes, ageing and longevity in humans: Problems, advantages and perspectives. Free Radic Res 2006;40:1303–1323.
6. Franceschi C, Motta L, Motta M, Malaguarnera M, CapriM, Vasto S, Candore G, Caruso C; IMUSCE. The extreme longevity: The state of the art in Italy. Exp Gerontol 2008; 43:45–52.
7. Capri M, Salvioli S, Monti D, Caruso C, Candore G, Vasto S,Olivieri F, Marchegiani F, Sansoni P, Baggio G, Mari D, PassarinoG, De Benedictis G, Franceschi C. Human longevity within an evolutionary perspective: The peculiar paradigm of a post-reproductive genetics. Exp Gerontol 2008;43:53–60.
8. Beekman M, Blanche´ H, Perola M, Hervonen A, Bezrukov V, Sikora E, Flachsbart F, Christiansen L, De Craen AJ, Kirkwood TB, Rea IM, Poulain M, Robine JM, Valensin S, Stazi MA, Passarino G, Deiana L, Gonos ES, Paternoster L, Sørensen TI, Tan Q, Helmer Q, van den Akker EB, Deelen J, Martella F, Cordell HJ, Ayers KL, Vaupel JW, To¨rnwall O, Johnson TE, Schreiber S, Lathrop M, Skytthe A, Westendorp RG, Christensen K, Gampe J, Nebel A, Houwing-Duistermaat JJ, Slagboom PE, Franceschi C; GEHA Consortium. Genome-wide linkage analysis for human longevity: Genetics of Healthy Aging Study. Aging Cell 2013;12:184–193.
9. Di Bona D, Accardi G, Virruso C, Candore G, Caruso C.Association between genetic variations in the insulin/insulinlike growth factor (IGF-1) signaling pathway and longevity: A systematic review and meta-analysis. Curr Vasc Pharmacol 2013; Dec 18. [Epub ahead of print] 10. Kuro-o M. Klotho and aging. Biochim Biophys Acta 2009; 1790:1049–1058.
11. Wang Y, Sun Z. Current understanding of klotho. AgeingRes Rev 2009;8:43–51.
12. Arking DE, Krebsova A, Macek M Sr, Macek M Jr, ArkingA, Mian IS, Fried L, Hamosh A, Dey S, McIntosh I, Dietz HC. Association of human aging with a functional variant of klotho. Proc Natl Acad Sci USA 2002;99:856–861.
13. Novelli V, Viviani Anselmi C, Roncarati R, Guffanti G,Malovini A, Piluso G, Puca AA. Lack of replication of genetic associations with human longevity. Biogerontology 2008;9:85–92.
14. Invidia L, Salvioli S, Altilia S, Pierini M, Panourgia MP,Monti D, De Rango F, Passarino G, Franceschi C. The frequency of Klotho KL-VS polymorphism in a large Italian population, from young subjects to centenarians, suggests the presence of specific time windows for its effect. Biogerontology 2010;11:67–73.
15. Majumdar V, Nagaraja D, Christopher R. Association ofthe functional KL-VS variant of Klotho gene with earlyonset ischemic stroke. Biochem Biophys Res Commun 2010;403:412–416.
16. DerSimonian R, Laird N. Meta-analysis in clinical trials.Controlled Clin Trials 1986;7:177–188.
17. Arking DE, Becker DM, Yanek LR, Fallin D, Judge DP,Moy TF, Becker LC, Dietz HC. KLOTHO allele status and the risk of early-onset occult coronary artery disease. Am J Hum Genet 2003;72:1154–1161
18. Arking DE, Atzmon G, Arking A, Barzilai N, Dietz HC.Association between a functional variant of the KLOTHO gene and high-density lipoprotein cholesterol, blood pressure, stroke, and longevity. Circ Res 2005;96:412–418
19. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A,Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Suppression of aging in mice by the hormone Klotho. Science. 2005;309:1829–1833
20. Ziv E, Hu D. Genetic variation in insulin/IGF-1 signalingpathways and longevity. Ageing Res Rev 2011;10:201–204.

Address correspondence to:
Danilo Di Bona
U.O. di Medicina Trasfusionale

AOUP Paolo Giaccone
Via del Vespro 129
90127 PalermoI
Italy

E-mail: danilodibona@yahoo.it

Received: October 26, 2013
Accepted: October 26, 2013

1.6 SHIP2: A "NEW" INSULIN PATHWAY TARGET FOR AGEING RESEARCH

REJUVENATION RESEARCH
Volume 17, Number 2, 2014

DOI: 10.1089/rej.2013.1541

SHIP2: A "NEW" Insulin Pathway Target for Aging Research

Giulia Accardi,[1] Claudia Virruso,[1] Carmela Rita Balistreri,[1] Fabrizio Emanuele,[2] Federico Licastro,[3] Roberto Monastero,[4] Elisa Porcellini,[3] Sonya Vasto,[5] Salvatore Verga,[2] Calogero Caruso,[1] and Giuseppina Candore[1]

Abstract

Strong evidence suggests that systemic inflammation and central adiposity contribute to and perpetuate metabolic syndrome. All of these alterations predispose individuals to type 2 diabetes mellitus (T2DM), cardiovascular disease, as well as Alzheimer's disease (AD), all characterized by chronic inflammatory status. On the other hand, extensive abnormalities in insulin and insulin-like growth factor I (IGF-I) and IGF-II signaling mechanisms in brains with AD have been demonstrated, suggesting that AD could be a third form of diabetes. The Src homology domain-containing inositol 5-phosphatase 2 (SHIP2) has an important role in the insulin pathway because its over-expression causes impairment of insulin/IGF-1 signaling. Because some single-nucleotide polymorphisms (SNP) of the gene encoding SHIP2 were significantly associated in T2DM patients with metabolic syndrome and some related conditions, we decided to conduct a case–control study on this gene, analyzing AD and T2DM subjects as cases and young, old, and centenarians as controls. Our results suggest a putative correlation between the the rs144989913 SNP and aging, both successful and unsuccessful, rather than age-related diseases. Because this SNP is an insertion/deletion of 28 bp, it might cause an alteration in SHIP2 expression. It is noteworthy that SHIP2 has been demonstrated to be a potent negative regulator of insulin signaling and insulin sensitivity. Many studies demonstrated the association of the insulin/IGF1 pathway with aging and longevity, so it is tempting to speculate that the found association with SHIP2 and aging might depend on its effect on the insulin/IGF-1 pathway.

Introduction

AGING IS AN INELUCTABLE process resulting from the interaction among genetic, epigenetic, stochastic, and lifestyle factors.[1,2] However, *in vivo* studies in model animals demonstrate that single genetic mutations are able to modulate life span. The insulin-like growth factor-I (IGF-I) pathway seems to be correlated to human life span, and its homologs are closely conserved in the main experimental models such as yeast, nematode, and fruit fly in which mutations in genes encoding proteins involved in this pathway affect life span.[3]

Insulin is the most potent anabolic hormone and is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis. Insulin resistance (IR) reflects impairments in the insulin signaling pathway, but the molecular mechanisms implicated are not so clear, although the inflammatory process is involved. IR is one of the features of metabolic syndrome, a pre-diabetic status.[4,5]

Interestingly, strong evidence suggests that systemic inflammation and central adiposity contribute to and perpetuate metabolic syndrome. All of these alterations predispose individuals to type 2 diabetes mellitus (T2DM), cardiovascular disease, as well as Alzheimer's disease (AD), all characterized by chronic inflammatory status.[6–12] In 2005, a group of American scientists hypothesized that AD could be a third form of diabetes. They demonstrated extensive abnormalities in insulin and IGF-I and IGF-II signaling mechanisms in brains with AD, showing that although each of the corresponding growth factors is normally made in central nervous system neurons, the expression levels are markedly reduced in AD.[13]

[1]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies and [2]Clinical Nutrition Unit, Biomedical Department of Internal and Specialty Medicine, University of Palermo, Palermo, Italy.
[3]Department of Experimental Diagnostic and Specialty Medicine, University of Bologna, Bologna Italy.
[4]Department of Experimental Biomedicine and Clinical Neurosciences and [5]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy.

Today, evidence demonstrates the presence of IR in subjects with neurodegeneration, such as AD or Parkinson's patients.[14] AD, the most common form of dementia, is characterized by accumulation of senile plaques constituted by deposits of the abnormal amyloid protein (Aβ40–42 amino acids) and neurofibrillary tangles originating from hyperphosphorylation of microtubular tau protein. The amyloid hypothesis is not unique for the pathogenesis of AD. Indeed, different pathophysiological theories exist that focus attention on inflammation, vascular changes, and metabolic disorders. The most plausible hypothesis is that all of these theories are not mutually exclusive and could be taken together. Actually, inflammation plays a relevant role in both vascular lesions and metabolic disorders and could be the link between AD and T2DM.[15–17] Moreover, some authors proposed the concept of "metabolic cognitive syndrome" based on the co-occurrence of AD and metabolic syndrome. Indeed, dementia and metabolic syndrome present some overlaps both in predisposition factors, such as diet, smoking, socio-economic status, and lifestyle, and in altered signaling cascades, *i.e.*, nutrient-sensing pathways such as the insulin pathway.[18]

The Src homology domain-containing inositol 5-phosphatase 2 (SHIP2), has an important role in the insulin pathway. It leads to the activation of AKT, acting on glycogen synthase kinase-3 (GSK3) (Fig. 1).[19,20] Dysregulation of GSK3 activity determines neuronal cell death, hyperphosphorylation of tau protein, and the production of amyloid protein with an involvement in the neuropathology of AD.[21,22] Many studies underline the role of SHIP2 as a probable negative regulator of insulin signaling.[19,23–25] A study conducted by Kaisaki et al. in T2DM subjects demonstrated a significant association between single-nucleotide polymorphisms (SNPs) of INPPL1 (rs2276047, rs9886, and rs144989913) and metabolic syndrome or correlated features,[26] a finding partly confirmed by another study.[27] Moreover, a study conducted in non-T2DM subjects with hypertension (one of the features of metabolic syndrome previously associated with the SNPs), found no association, identifying the T2DM as condition probably necessary for the association.[28]

Starting from all of these studies and observations, we decided to conduct a case–control study on this gene, analyzing AD and T2DM subjects as cases and young, old, and centenarians as controls, with the aim to strengthen the association between the above-mentioned age-related diseases. In particular, we studied two polymorphisms of INPPL1 (which encodes inositol polyphosphate-5 phosphatase-like 1), rs9886 and the rs144989913.

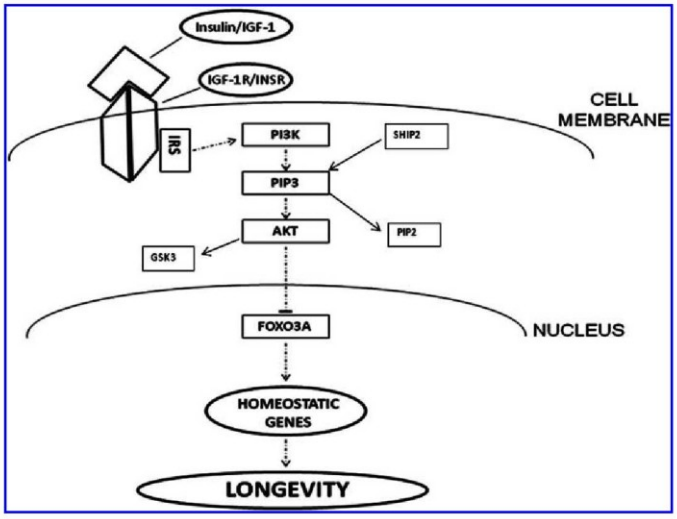


FIG. 1. Insulin-like growth factor I (IGF-1) pathway and Src homology domain-containing inositol 5-phosphatase 2 (SHIP2) action. This signaling pathway has a critical role in the determination of longevity. The bond of insulin/IGF-1 to the specific receptor (IGF-1R/INSR) activates the phosphatidylinositol-3′-kinase (PI3K) through the insulin-related substrate (IRS). It leads to the activation of AKT, through phosphatidylinositol(3,4,5)-trisphosphate (PIP3), which, in turn, inhibits Forkhead box O3A (FOXO3A). FOXO3A acts as transcription factor, activating the expression of many homeostatic genes. In the meantime, the downstream signal activated from PIP3 leads to the activation of AKT/protein kinase B (PKB), which phosphorylates and inactivates the glycogen synthase kinase 3 (GSK3). SHIP2 acts on the substrate lipid secondary messenger PIP3 to produce phosphatidylinositol 3,4-diphosphate (PIP2). Thus, SHIP2 is an antagonist of PI3K that phosphorylates PIP2 to obtain PIP3, attenuating the PI3K-mediated insulin signaling pathway.

Material and Methods

Sample collection

Informed consent was obtained from all cases of T2DM or guardians of AD patients and controls according to Italian law. In all, we collected 468 whole blood samples in EDTA Vacutainers.

Specifically, we enrolled 127 unrelated young subjects (mean age 35) randomly selected from blood donors and 105 old people (mean age 72), as controls. They were checked and judged to be in good health on the basis of their clinical history and on blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C-reactive protein, liver function test, iron, proteins, cholesterol, and triglycerides). Moreover, we selected 119 subjects probably affected by AD (mean age 77) as cases. AD patients were diagnosed according to standard clinical procedures and followed the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) and *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* (DSM-IIIR) criteria. Cognitive performance and alterations were measured according to the Mini-Mental State Evaluation and the Global Deterioration Scale. These cases were defined as sporadic because their family history did not mention any first-degree relative with dementia.[29,30] A total of 117 subjects affected by T2DM (mean age 68), diagnosed according to joint criteria of American Diabetes Association, the European Association for the Study of Diabetes, and the International Diabetes Federation, were also enrolled as cases. Moreover, we analyzed 20 DNA samples of centenarians belonging to our DNA bank.

Genetic analysis

Peripheral whole blood samples were collected and genomic DNA was extracted from leukocytes by a commercial kit. We genotyped the SNP rs144989913, which is an insertion/deletion (I/D) of 28 base pairs, by classic PCR and rs9886 by amplification-refractory mutation system (ARMS PCR). The size separation was conducted using agarose gel electrophoresis (3%).

Statistical analysis

The data were tested by a chi-squared test for the goodness of fit between the observed and expected genotype frequencies according to the Hardy–Weinberg equilibrium (HWE). Differences in allele and genotypic frequencies of the two SNPs among the groups were evaluated by gene count and the chi-squared test.

Results

A total of 488 individuals have been genotyped for the two SNPs. The frequencies of the genotypes of all SNPs under investigation, both in cases and controls, are in HWE. Table 1 shows the genotype and allele frequencies in all subjects of the two SNPs of INPPL1. We did not find any association for the rs9886 polymorphism, both for genotypic and allelic frequencies (data not shown). The distribution of the rs144989913 genotype between T2DM and young, old and young, and young and centenarians is significantly different. The frequency of the heterozygous genotype was increased in T2DM and AD patients as well as in old and centenarians with respect to young subjects. According to the genotype, a significant difference in the rs144989913 allele frequencies between T2DM and young, AD and young, old and young, and young and centenarians was observed. There were no significant differences for genotype and allele frequencies between T2DM and old or centenarians, AD and old, or centenarians and old and centenarians. Focusing on allelic frequencies of the D allele of rs144989913, we highlighted, with a 3×2 table, a growing significant increase ($p=0.0016$) of D with increasing age (young = 0.39; old = 0.11; centenarians = 0.15).

Gender analysis demonstrated that the significant difference in the rs144989913 genotypic and allele frequencies between T2DM and young, AD and young, and old and

TABLE 1. RS144999813 GENETIC DISTRIBUTION AND ALLELE FREQUENCY FOR CASES REPRESENTED BY ALZHEIMER DISEASE SUBJECTS AND TYPE 2 DIABETES MELLITUS SUBJECTS AND CONTROLS REPRESENTED BY YOUNG, OLD, AND CENTENARIANS SUBJECTS AND ASSOCIATION OF THE RS144999813 BETWEEN CASES AND CONTROLS AND YOUNG AND AGED PEOPLE

Genotype	*T2DM* (n = 117) *(A)*	*AD* (n = 119) *(B)*	*Young* (n = 127) *(C)*	*Old* (n = 105) *(D)*	*Centenarian* (n = 20) *(E)*
I/I	97 (0.83)	97 (0.81)	117 (0.92)	81 (0.77)	14 (0.70)
I/D	20 (0.17)	22 (0.19)	10 (0.08)	24 (0.13)	6 (0.30)
D/D	0	0	0	0	0
P	AvsC = 0.028*	BvsC = 0.013*	CvsD = 0.0013*	DvsE = ns*	
	AvsD = ns*	BvsD = ns*	CvsE = 0.0031*		
	AvsE = ns*	BvsE = ns*			
ALLELE					
I	214 (0.91)	216 (0.91)	244 (0.96)	186 (0.88)	34 (0.85)
D	20 (0.09)	22 (0.09)	10 (0.039)	24 (0.11)	6 (0.15)
P	AvsC = 0.034**	BvsC = 0.017**	CvsD = 0.0020**	DvsE = ns**	
	AvsD = ns**	BvsD = ns**	CvsE = 0.004**		
	AvsE = ns**	BvsE = ns**			

*The significance of the different genotype distribution among groups was calculated by chi-squared test (3×2 table).
**The significance of the different allele distribution among groups was calculated by chi-squared test (2×2 table).
T2DM, type 2 diabetes mellitus; AD, Alzheimer's disease; I, insertion; D, deletion; ns, not significant.

young are present only in males rather than in both males and females (data not shown). Due to small number of centenarians, we could not study the gender effect in this population.

Discussion

Our study concerned the association between INPPL1 SNPs and age-related diseases, aging, and longevity. The results indicate a significant association of the rs144989913 polymorphism with both successful and unsuccessful aging. In a previous report, rs9886 and rs144989913 were shown to be associated, in haplotype, with rs2276047, to hypertension, obesity, metabolic syndrome, and T2DM, but no association with only rs2276047 was shown.[26] Thus, we exclusively analyzed the two above-mentioned SNPs but we obtained significant results only for rs144989913.

Both genotypic and allelic frequencies of rs144989913 showed significant association of this SNP between young and old in general, rather than between elderly and the specific age-related diseases. The frequency of the D allele increased from young to centenarians. Therefore, in a further step, the life expectancy should be analyzed in aged patients with the D allele in comparison with the I allele. Moreover, the specific association with males in not surprising because it has been claimed that males and females follow different strategies to attain longevity, and several case–control studies have been positive only in males.[31–33]

Concerning the function of SHIP2, it is noteworthy that its acts inside the signaling cascade of insulin, hence its alteration in terms of function and expression may cause insulin pathway impairment. Indeed, *in vivo* studies, demonstrated that SHIP2 is a potent negative regulator of insulin signaling and insulin sensitivity.[18,22–24] Many studies have demonstrated the association of the insulin/IGF1 pathway with aging and longevity. The replication of specific results in model organisms led to conducting studies also in human.[3,33] It is tempting to speculate that rs144989913 alleles may differently influence gene expression because they consist of a variation of 28 base pairs. They may differently modulate the insulin pathway involved in aging and longevity, hence functional studies are mandatory to confirm this suggestion.

In conclusion, our results are only a small contribution to aging research, but they do represent the first study that couples INPPL1/SHIP2 and aging. INPPL1 might be a "new" interesting gene in aging research, and this study represents the first step.

Acknowledgments

This work was supported by the authors' respective institutions (ex 60% to G.C. and C.C.). G.A. and C.V. are PhD students (Tutor G.C., Supervisor C.C.), and this paper is submitted in partial fulfillment of their PhD degree.

Author Disclosure Statement

The authors have no conflict of interest.

References

1. Caruso C, Passarino G, Puca A, Scapagnini G. "Positive biology": The centenarian lesson. Immun Ageing 2012;9:5.
2. Montesanto A, Dato S, Bellizzi D, Rose G, Passarino G. Epidemiological, genetic and epigenetic aspects of the research on healthy ageing and longevity. Immun Ageing 2012;9:6.
3. Ziv E, Hu D. Genetic variation in insulin/IGF-1 signaling pathways and longevity. Ageing Res Rev 2011;10:201–204.
4. Wilcox G. Insulin and insulin resistance. Clin Biochem Rev 2005;26:19–39.
5. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006;116:1793–1801.
6. Elks CM, Francis J. Central adiposity, systemic inflammation, and the metabolic syndrome. Curr Hypertens Rep 2010;12:99–104.
7. Ahtiluoto S, Polvikoski T, Peltonen M, Solomon A, Tuomilehto J, Winblad B, Sulkava R, Kivipelto M. Diabetes, Alzheimer disease, and vascular dementia: A population-based neuropathologic study. Neurology 2010;75:1195–1202.
8. Luchsinger JA. Insulin resistance, type 2 diabetes, and AD: Cerebrovascular disease or neurodegeneration? Neurology 2010;75:758–759.
9. Sanz C, Andrieu S, Sinclair A, Hanaire H, Vellas B; REAL.FR Study Group. Diabetes is associated with a slower rate of cognitive decline in Alzheimer disease. Neurology 2009;73:1359–1366.
10. Vanhanen M, Koivisto K, Moilanen L, Helkala EL, Hanninen T, Soininen H, Kervinen K, Kesaniemi YA, Laakso M, Kuusisto J. Association of metabolic syndrome with Alzheimer disease: A population-based study. Neurology 2006;67:843–847.
11. Razay G, Vreugdenhil A, Wilcock G. The metabolic syndrome and Alzheimer disease. Arch Neurol 2007;64:93–96.
12. Haslam DW, James WP. Obesity. Lancet 2005;366:1197–1209.
13. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? J Alzheimer Dis 2005;7:63–80.
14. Domínguez RO, Pagano MA, Marschoff ER, González SE, Repetto MG, Serra JA. Alzheimer disease and cognitive impairment associated with diabetes mellitus type 2: Associations and a hypothesis. Neurologia, Barcelona, Spain, 2013;S0213–4853.
15. De la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. Lancet Neurol 2004;3:184–190.
16. Milionis HJ, Florentin M, Giannopoulos S. Metabolic syndrome and Alzheimer's disease: A link to a vascular hypothesis? CNS Spectr 2008;13:606–613.
17. Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Di Bona D, Duro G, Lio D, Matranga D, Pellicano' M, Rizzo C, Scapagnini G, Vasto S. Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in Alzheimer disease: Therapeutic implications. Rejuvenation Res 2010;13:301–313.
18. Frisardi V, Solfrizzi V, Capurso C, Imbimbo BP, Vendemiale G, Seripa D, Pilotto A, Panza F. Is insulin resistant brain state a central feature of the metabolic-cognitive syndrome? J. Alzheimers Dis 2010;21:57–63.
19. Dyson JM, Kong AM, Wiradjaja F, Astle MV, Gurung R, Mitchell CA. The SH2 domain containing inositol polyphosphate 5 phosphatase-2: SHIP2. Int J Biochem Cell Biol 2005;37:2260–2265.

20. Plum L, Schubert M, Bruning JC. The role of insulin receptor signaling in the brain. Trends Endocrino Metab 2005;16:59–65.
21. Lucas JJ, Hernández F, Gómez-Ramos P, Morán MA, Hen R, Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J 2001 15;20:27–39.
22. Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 2004;29:95–102.
23. Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L, Schurmans S. The lipid phosphatase SHIP2 controls insulin sensitivity. Nature 2001;409:92–97.
24. Soeda Y, Tsuneki H, Muranaka H, Mori N, Hosoh S, Ichihara Y, Kagawa S, Wang X, Toyooka N, Takamura Y, Uwano T, Nishijo H, Wada T, Sasaoka T. The inositol phosphatase SHIP2 negatively regulates insulin/IGF-I actions implicated in neuroprotection and memory function in mouse brain. Mol Endocrinol 2010;24:1965–1977.
25. Hori H, Sasaoka T, Ishihara H, Wada T, Murakami S, Ishiki M, Kobayashi M. Association of SH2-containing inositol phosphatase 2 with the insulin resistance of diabetic db/db mice. Diabetes 2002;51:2387–2394.
26. Kaisaki PJ, Delepine M,Woon PY, Sebag-Montefiore L, Wilder SP,Menzel S, Vionnet N,Marion E, Riveline JP, Charpentier G, Schurmans S, Levy JC, Lathrop M, Farrall M, Gauguier D. Polymorphisms in type II SH2 domain-containing inositol 5-phosphatase (INPPL1, SHIP2) are associated with physiological abnormalities of the metabolic syndrome. Diabetes 2004;53:1900–1904.
27. Kagawa S, Sasaoka T, Yaguchi S, Ishihara H, Tsuneki H, Murakami S, Fukui K, Wada T, Kobayashi S, Kimura I, Kobayashi M. Impact of SRC homology 2-containing inositol 5-phosphatase 2 gene polymorphisms detected in a Japanese population on insulin signaling. J Clin Endocrinol Metab 2005;90:2911–2919.
28. Marçano AC, Burke B, Gungadoo J, Wallace C, Kaisaki PJ, Woon PY, Farrall M, Clayton D, Brown M, Dominiczak A, Connell JM, Webster J, Lathrop M, Caulfield M, Samani N, Gauguier D, Munroe PB. Genetic association analysis of inositol polyphosphate phosphatase-like 1 (INPPL1, SHIP2) variants with essential hypertension. J Med Genet 2007;44:603–605.
29. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Service Task Force on Alzheimer's Disease. Neurology 1984;34: 939–944.
30. American Psychiatric Association. *American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders* (DSM-IIIR), 3rd revised ed. Washington DC: American Psychiatric Association, 1987.
31. Candore G, Caruso C, Colonna-Romano G. Inflammation, genetic background and longevity. Biogerontology 2010; 11:565–573.
32. Capri M, Salvioli S, Monti D, Caruso C, Candore G, Vasto S, Olivieri F, Marchegiani F, Sansoni P, Baggio G, Mari D, Passarino G, De Benedictis G, Franceschi C. Human longevity within an evolutionary perspective: The peculiar paradigm of a post-reproductive genetics. Exp Gerontol 2008;43:53–60.
33. Di Bona D, Accardi G, Virruso C, Candore G, Caruso C. Association between genetic variations in the insulin/insulin-like growth factor (IGF-1) signalling pathway and longevity: A systematic review and meta-analysis. Curr Vasc Pharmacol Dec 18. [Epub ahead of print]

Address correspondence to:
Giulia Accardi
Immunosenescence Unit
Department of Pathobiology
and Medical and Forensic Biotechnologies
University of Palermo
Corso Tukory 211
90134 Palermo
Italy

E-mail: giuliabio@gmail.com

Received: December 8, 2013
Accepted: December 8, 2013

1.7 DISCUSSION

A growing amount of evidences shows that dietary intervention and genetic alterations in gene encoding proteins that take part in metabolic nutrient-sensing pathways can modulate lifespan (*Bonafè et al 2003; Suh et al 2008; Willcox et al 2008; Albani et al 2009; Anselmi et al 2009; Flachsbart et al 2009; Soerensen et al 2010; Ziv et al 2011*). It depends on the hyper o ipo activation of these signaling due to genetic mutations that under or over express regulative molecules leading to different expression of homeostatic genes.

During evolution, this pathway has diverged from a single receptor in invertebrates to multiple receptors and more complicated pathways and regulatory networks in mammals.

The first signaling cascade associated with ageing and longevity was the insulin/IGF-1 pathway in *C. elegans.* It was shown that mutation that reduce the *daf-2* function, orthologue of IGF-1 receptor, and mutation in *age-1*, homologue of PI3K, lead to both increased life span and stress resistance (*Dorman et al 1995; Apfeld et al 1998*).

Also in mice and in primates the modulation of this pathway can extend life-span and delay age-related pathologies leading to the conclusion that these associations are evolutionary conserved (*Bartke 2005; Anderson et al 2009; Fontana et al 2010*).

Our results, obtained from meta-analyses and candidate gene approach, support data previous shown for the role of specific SNPs in ageing and longevity.

Among others, FOXO3A probably represents one of the genes that more influence longevity, association observed in different ethnic

populations. Moreover, a multitude of studies in *C. elegans* support its role. *Daf-16* is the homologue of FOXO in the nematode. Evidences demonstrated that it protects cells from oxidative stress that constitutes a nerve centre in ageing process, increasing life-span (*Kenyon 2005*). Daf-16 is a TF that modulates the expression of SOD2, acting as free radical scavenger (*Honda et al 1999*). It seems that the role of FOXO3A in human might be the same, acting as a TF on multiple homeostatic genes in response to decreased insulin/IGF-1 signaling and consequently increasing life-span (*Ziv et al 2011*). Interestingly, other genes that increase life-span, i.e. the enzymes histone deacetylase sirtuins, interact with FOXO. In particular, SIRT1 deacetylates FOXO3A and modulates its response to oxidative stress (*Salminen et al 2009*).

Our studies confirm previous studies about the association between FOXO3A, IGF-1R and longevity but no association was reported between IGF-1 and SIRT1 (for the analyzed SNPs), although, for IGF-1, its SNPs could affect the serum levels, known to modulate ageing and longevity and higher circulating levels of IGF-1 have also been associated with longer leukocyte telomere length, a key biomarker of human ageing, in healthy subjects (*Barbieri et al 2009*).

This would be consistent with the hypothesis that most longevity genes have modest or small effect sizes. It is also possible that small sample size and the remarkable heterogeneity often observed in the populations included in the different studies, in terms of age and ethnicity of both control and cases groups, limited our detection ability. Another explanation could be that these contrasting results are due to the insulin/IGF-1 paradox (*Koshiyama 2012*).

Moreover, we observed sex-specific differences in the association of the genetic variation with survival during old age. In particular, about FOXO3A the significant association with longevity was observed specifically when only males were included in the analysis. Also for the rs144989913, SNP of INPPL1, we obtain a specific association in males. This is not surprising because, as we discussed in our paper (*Caruso et al 2013*), it has been claimed that males and females follow different strategies to attain longevity and several association studies have been positive only in males (*Capri et al 2008*). The reason are obviously multifactorial, with a socio-cultural component that can be distinguish from biological trait linked to longevity. In some cases our result became positive only in centenarians. Also this result is not surprising, because centenarians represent the survival tail of the population (*Caruso et al 2012*).

Our second meta-analysis on the association between KLOTHO KL-VS variant (stretch that contains six polymorphisms in linkage disequilibrium) and lifespan indicated a significant association of the variant with healthy ageing and longevity, despite the serious limitations of the study. This association is limited to KL-VS heterozygous people because the KL-VS homozygous undergoes to a detrimental effect of the polymorphism indicating a possible association mechanism not related to the gene dose. It should be noted that in one study the genetic effect was shown only in one population, suggesting that genetic or environmental factors could influence the observed effect. These contrasting results could be linked to the reason mentioned above that partially mask the true genetic effect.

However, cross-sectional and prospective studies confirm a genetic model in which the KL-VS allele confers a heterozygous advantage in conjunction with a marked homozygous disadvantage with low levels of HDL cholesterol, high systolic blood pressure, increased risk of stroke and early onset coronary artery disease, and mortality (*Arking et al 2003; Arking et al 2005*).

Coming back to the crucial nodes upstream to insulin/IGF-1 pathway, certainly we have to highlight the importance of the second messenger PIP3. Indeed, from this molecules start the signal that activate the kinase AKT, involved in many crucial cellular activity. To regulate PIP3 level, the cell uses a kinase and a phosphatase, PI3K and phosphatase and tensin homolog and SHIP2. Since the role of SHIP2 is the less clear, we analyzed the association of some its SNPs with age-related diseases. Variation in SHIP2 levels may cause insulin pathway impairment. Indeed, *in vivo* studies, demonstrated that SHIP2 is a potent negative regulator of insulin signaling and insulin sensitivity (*Clement et al 2001; Soeda et al 2010*). Since the rs144989913 alleles is an insertion/deletion of 28 base pairs, it is tempting to speculate that might differently influence gene expression of INPPL1. Of course functional studies are mandatory to confirm this suggestion but the increased frequency of the deletion allele from young to centenarians may be due to a different expression of SHIP2 that reduce the insulin signaling possibly favoring longevity.

Summarizing, the studied allelic variants reduce the insulin/IGF-1 signaling, hence, we agreed that a down regulation of this pathway can increase lifespan in human leading to healthy lifespan.

In rodents, both dietary restriction (DR) and mutations in nutrient and growth signaling pathways can extend longevity by 30-50% and lower the incidence of age-related loss of function and disease, including tumors and neurodegeneration. DR also increases "healthspan" and protects against diabetes, cancer, and cardiovascular disease in rhesus monkeys, and in humans it causes changes that protect against these age-related pathologies (*Omodei et al 2011*).

Pharmaceutical interventions that directly regulate nutrient/sensing pathways in adults could improve health and prevent age-related diseases, but before drugs can be considered for chronic administration, it requires large investments. Drastic dietary restriction could cause adverse effects especially in old and frail subjects. Therefore, an alternative approach might be a close adherence to MD that includes an healthy lifestyle. MD is a real culture that has been reported to contribute to better health and quality of life in the Mediterranean countries. Especially in Sicily and Sardinia, where many longevous people exist, this "cultural habit" is common. The Elderly Prospective Cohort Study identified a reduced overall mortality among old people that live in a "Mediterranean way" and in particular in that people consuming monounsaturated (MUFA) fatty acid instead of satured (*Trichopoulou et al 2005; Bürkle et al 2007*).

Meta-analyses of prospective cohort studies demonstrate that the adherence to MD can significantly decreases the risk of mortality from CVDs (in particular from coronary heart disease) and the incidence of PD, AD and cancer (*Sofi et al 2008; Estruch et al 2013*). It is well established that the pathophysiology of common age-related disease is associated with chronic inflammation and oxidative stress and that

LDL oxidation is one of the major risk factor for the development of CVDs (*Candore et al 2010*). Extra virgin olive oil (EVOO), the main source of polyphenols in MD, is composed by MUFA, mainly oleic acid, that reduces LDL cholesterol levels in comparison with saturated fats. *In vivo,* three mechanistic studies have shown that EVOO phenolic compounds are able to bind to LDL and this may increase the resistance to oxidation. Furthermore, the inhibition of NF-kB pathway activation by polyphenols could explain part of its anti-inflammatory properties. EVOO also contains carotenoids, sterols, lycopene, and hydrophilic phenolics (oleuropein, oleocanthal, hydroxytyrosol and tyrosol), all bioactive compounds (*Pitozzi et al 2012*). *In vivo* and *in vitro* research has suggested that the dietary intake of EVOO with high polyphenols content may attenuate inflammatory response and therefore reduce the risk of chronic inflammatory diseases (*Corona et al 2009; Konstantinidou et al 2010; Khymenets et al 2009*). Oleuropein is a radical scavenger that blocks the LDL oxidation (*de la Torre-Carbot et al 2007*). Moreover, *in vitro* studies demonstrated the Ibuprofen–like activity of oleocanthal and hydroxytyrosol carried out the inhibition of the cyclooxygenases 1 and 2, responsible for prostaglandin production, in a dose-dependent manner (*Beauchamp et al 2005; Gonzalez-Santiago et al 2010*). The nutraceutical properties of EVOO are summarized in the review "Nutraceuticals properties of Extra virgin Olive oil (EVOO): a natural remedy for age-related diseases?", in the next session.

1.8 NUTRACEUTICAL PROPERTIES OF EXTRA VIRGIN OLIVE OIL (EVOO): A NATURAL REMEDY AGE-RELATED DISEASES?

REJUVENATION RESEARCH
Volume 17, Number 2, 2014

DOI: 10.1089/rej.2013.1532

Nutraceutical Properties of Extra-Virgin Olive Oil: A Natural Remedy for Age-Related Disease?

Claudia Virruso,[1] Giulia Accardi,[1] Giuseppina Colonna-Romano,[1] Giuseppina Candore,[1] Sonya Vasto,[2] and Calogero Caruso[1]

Abstract

The health benefits of the Mediterranean diet can be largely ascribed to the nutraceutical properties of extra-virgin olive oil (EVOO). Mono-unsaturated fatty acids and various phenolic compounds, such as oleocanthal, oleuropein, hydroxytyrosol, and tyrosol, are the main nutraceutical substances of EVOO. These substances have been suggested to have the ability to modulate aging-associated processes. In experimental models, it has been shown that EVOO with high concentrations of polyphenols has anti-inflammatory and anti-oxidant properties. Indeed, it was observed that hydroxytyrosol and oleocanthal inhibit the cyclooxygenases (COX-1 and −2) responsible for prostaglandin production; oleuropein is a radical scavenger that blocks the oxidation of low-density lipoproteins. Due to the relevance of olive oil in the economy of Sicily, our group has been funded to assess the nutraceutical properties of different kinds of olive oil. Indeed, the aim of the study is to evaluate effects of EVOOs, with low and high polyphenols content, on immuno-inflammatory and oxidative stress responses in young and old people. A further objective of our group is to evaluate effects of EVOO, with low and high polyphenol content, on the expression of genes encoding proteins that take part in the insulin/insulin-like growth factor-1 signaling pathway involved in longevity. The results of the study will be useful for producing olive oil enriched in nutraceutical properties that may be likely helpful in the prevention of age-related diseases.

Introduction

In recent years, researchers have been focused on the identification of functional food or nutraceuticals, which have been suggested to have an important effect in extending life span and, in general, improving health. The term "nutraceutical," coined by Stephen DeFelice in 1989, specifies a new generation of bioactive compounds of natural origin with effects on health. In particular, "nutraceuticals must not only supplement the diet but should also aid in the prevention and/or treatment of disease and/or disorder" and "are represented for use as a conventional food or as the sole item of meal or diet."[1]

Many studies show a higher life expectancy in Mediterranean populations in comparision with others countries of northern Europe. Meta-analysises of prospective cohort studies demonstrate that the adherence to a Mediterranean diet can significantly decrease the risk of mortality from cardiovascular diseases (CVD), incidence of Parkinson and Alzheimer diseases, and the incidence of mortality from cancer.[2] In particular, epidemiologic evidence shows that the Mediterranean diet mainly reduces the incidence of coronary heart disease.[3]

The Mediterranean diet is an eating pattern characterizing a lifestyle and culture that has been reported to contribute to better health and quality of life in the Mediterranean countries. It is traditionally characterized by plant foods (fruit, vegetables, legumes, wholemeal bread, and other forms of cereals, nuts, and seeds), fresh fruit, olive oil, dairy products (principally cheese and yogurt), and poultry consumed in low to moderate amounts (fish only by coast inhabitants), zero to four eggs consumed weekly, red meat consumed in very low amounts, and wine consumed in low to moderate amounts, normally with meals. Given that within Mediterranean countries cultural and religious differences exist that bring about diversity in food patterns, today the concept of "Mediterranean diets" is more commonly applied than that of a specific one. However, these diets prevail in the olive tree–growing areas in the countries of the Mediterranean basin, hence they are characterized by the use of olive oil as the principal source of fat.[4]

[1]Immunosenescence Unit, Department of Pathobiology and Medical and Forensic Biotechnologies, and [2]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy.

In particular, extra-virgin olive oil (EVOO) is thought to promote good health and longevity.[5] Extracted from olive fruits of *Olea europea*, EVOO is described as having a free acidity, expressed as oleic acid, of not more than 0.8 grams/100 grams and a peroxide value of less than 20 milli-equivalents of oxygen. It must be produced entirely by mechanical means without the use of any solvents and under temperatures that will not degrade the oil (less than 30°C).[6] It is composed of monounsaturated fatty acids, mainly oleic acid, that reduce low-density lipoprotein cholesterol (LDL-C) levels in comparison with saturated fats. Olive oil also contains carotenoids, sterols, lycopene, and hydrophilic phenolics (oleuropein, oleocanthal, hydroxytyrosol, and tyrosol), which represent the most abundant family of bioactive EVOO compounds.[7] The mechanical process for the olive oil production, cold-press, preserves both the chemical nature of the oil and the natural anti-oxidants that the olive tree produces in response to environmental stress.[8] These substances have been suggested to have the ability to modulate aging-related processes. Indeed, it is well established that the pathophisiology of common age-related disease such as cancer, CVD, arthritis, and neurodegenerative disease are associated with chronic inflammation and oxidative stress.[9] In experimental models, it has been shown that EVOO with high concentration of polyphenols has anti-inflammatory and anti-oxidant properties.[10]

Olive Oil phenolic Compounds: Anti-Oxidant and Anti-Inflammatory Properties

To date, more of 36 phenolic compounds have been identified in EVOO in different concentrations and compositions (0.02–600 mg/kg), depending on the variety, the growing region, the agricultural techniques, the maturity at harvest, and the processing of the olive fruit.[11] These compounds are bioavailable and are thus absorbed, metabolized, distributed, and eliminated. In the case of poor absorption, it has been suggested that these compounds may exert local anti-oxidant activity in the gastrointestinal tract for their free-radical scavenging capacity.[12]

It is well known that an excess of free radicals can cause oxidative damage to biomolecules (lipids and DNA), increasing the risk of developing numerous chronic diseases.[13] For example, LDL oxidation is considered a major risk factor for the development of atherosclerosis and cardiovascular disease because it induces plaque formation within the arterial wall.

In vivo, three mechanistic studies have further shown that EVOO phenolic compounds are able to bind to LDL and this may increase resistance to oxidation; oleuropein is a radical scavenger that blocks the LDL oxidation.[14–16] Other studies have shown beneficial effects of olive oil phenolic compounds on other markers of oxidative stress. In human subjects, the intake of a phenols-enriched (400 mg/kg) olive oil significantly lowered F_2-isoprostane levels, compared to an olive oil poor in phenolic compounds (80 mg/kg). F_2-isoprostanes are producted consequently by a peroxidation of arachidonic acid, a common membrane constituent. Phenol-rich EVOO modulates the balance between gluthathione and gluthathione reductase and increases the amount of gluthathione peroxidase in erythrocytes with a beneficial effects.[17,18]

In vivo and *in vitro* research has suggested that the dietary intake of EVOO with high polyphenols content may attenuate inflammatory response and therefore reduce the risk of chronic inflammatory diseases.[19–21] *In vitro*, oleocanthal as well as hydroxytyrosol, have been shown to have ibuprofen-like activity. Indeed, they inhibit the cyclooxygenases 1 and 2 (COX-1 and 2), responsible for prostaglandin production, in a dose-dependent manner.[22,23]

This compound has potent pharmacological actions in attenuating inflammatory mediators such as inducible nitric oxide synthase (iNOS), which plays a role in the pathogenesis of degenerative diseases.[24] Oleocanthal seems also to possess anti-proliferatory effects in human breast and prostate cancer cell lines[25]; moreover, it promotes cell apoptosis by activating caspase-3 and induces fragmentation of DNA in HT-29 cells derived from human colon adenocarcinoma.[26]

Furthermore, the inhibition of the nuclear factor-κB (NF-κB) pathway activation by polyphenols could explain part of anti-inflammatory properties of EVOO. NF-κB induces the expression of a wide variety of genes active in inflammation that include pro-inflammatory cytokines, enzymes (iNOS), adhesion molecules, and acute-phase proteins. Thus, NF-κB is a suitable target to prevent or reduce an inflammatory response. A group of research has studied the effects of some polyphenols on NF-κB activation using human intestinal Caco-2 cells. Data indicate that some of the tested polyphenols were able to modulate this pathway by reducing the levels of the inhibitor-kB phosphorylated; by inhibiting the NF-κB induction initiated by the cytokines and lipopolysaccharide; and by reducing the secretion of the pro-inflammatory cytokine interleukin-8.[27]

Nutrigenomic Effects of Virgin Olive Oil Polyphenols

Nutrigenomics is a new field in the "omics" sciences and for this reason represents a specialized branch of post-genomics personalized medicine focusing on the interaction between food and the genome with an implication for both promoting health and preventing disease. Together with proteomics, nutrigenomics offers an advantage for understanding the interactions between the nutrients and protein translation, expression, and modification on the scale of the human proteome, as well as the role of hereditary factors in relation to food effects.[28]

At present, few data exist regarding the *in vivo* effect of the Mediterranean diet on human gene expression. Some of the gene expression-mediated mechanisms underlying the beneficial health effects of particular components of olive oil in humans have been already examined *in vitro* and in animal models. It has been shown that olive oil polyphenols inhibit the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) phosphorylation, resulting in downstream reduction in COX-2 expression.[29] These findings, obtained in cell culture or in animal models, are relevant for the knowledge of the relation between dietary components and gene expression, but are limited by the use of doses higher than those ingested in the human diet. The identification of peripheral blood mononuclear cells genes responding to EVOO consumption offers insight into the biological molecular

mechanisms underlying the benefits of EVOO on human health, particularly in prevention against age-related diseases. Accordingly, an altered expression of genes related to atherosclerosis development and progression has been demonstrated after 3 weeks of nutritional intervention with EVOO supplementation.[21]

Conclusions

Due to the relevance of olive oil in the economy of Sicily, our group has been funded to assess the nutraceutical properties of different kinds of EVOO. Indeed, the aim of the study is to evaluate effects of EVOOs, with low (L-EVOO) and high (H-EVOO) polyphenols content, on the immuno-inflammatory and oxidative stress responses in young and old people. Moreover, we will evaluate the expression of genes encoding proteins that take part in insulin/insulin-like growth factor-1 (IGF-1) signaling pathway, *i.e.*, IGF-1, IGF-1 receptor, forkhead box O (FOXO) 3A, and sirtuin 1. Indeed, many gene mutations, in particular single-nucleotide polymorphisms, associated with longevity or with increased life span were identified in gene-encoded proteins that take part in this metabolic pathway, as clearly demonstrated by our recent meta-analysis.[30] Due to a key role of FOXO3A in attaining healthy longevity, because it acts as transcription factor activating the expression of many homeostatic genes, we want to investigate the putative action, *in vivo* and *in vitro*, of EVOO on expression of this gene. The results of the study will be useful for producing olive oil enriched in nutraceutical properties that will likely be helpful in the prevention of age-related diseases.

Acknowledgments

This work was supported by PON DIMESA (Valorisation of typical products of the Mediterranean diet and their nutraceutical use to improve health). G.A. and C.V. are PhD students (Tutor G.C., Supervisor C.C.), and this paper is submitted in partial fulfillment of their PhD degree.

Author Disclosure Statement

No competing financial interests exist.

References

1. Kalra EK. Nutraceutical—definition and introduction. AAPS PharmSci 2003;5:E25.
2. Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. Adherence to Mediterranean diet and health status: Meta-analysis. Br Med J 2008;337:a1344.
3. Estruch R, Ros E, Salas-Salvadó J, Covas MI, Corella D, Arós F, Gómez-Gracia E, Ruiz-Gutiérrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pintó X, Basora J, Muñoz MA, Sorlí JV, Martínez JA, Martínez-González MA. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med 2013; 368:1279–1290.
4. Vasto S, Buscemi S, Barera A, Di Carlo M, Accardi G, Caruso C. Mediterranean diet and healthy aging—a Sicilian perspective. Gerontology (submitted).
5. Trichopoulou A. Traditional Mediterranean diet and longevity in the elderly: A review. Public Health Nutr 2004; 7:943–947.
6. Kiritsakis P, Kiritsakis AK, Lenart E, Willet WC, Hernandez RJ. *Olive Oil, from the Tree to the Table*, 2nd ed. Wiley-Blackwell: Hoboken, NJ.
7. Larsen LF, Jespersen J, Marckmann P. Are olive oil diets antithrombotic? Diets enriched with olive, rapeseed, or sunflower oil affect postprandial factor VII differently. Am J Clin Nutr. 1999;70:976–982.
8. Stark AH, Madar Z. Olive oil as a functional food: Epidemiology and nutritional approaches. Nutr Rev 2002; 60: 170–176.
9. Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Di Bona D, Duro G, Lio D, Matranga D, Pellicanò M, Rizzo C, Scapagnini G, Vasto S. Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in Alzheimer disease: Therapeutic implications. Rejuvenation Res 2010;13:301–313.
10. Pitozzi V, Jacomelli M, Catelan D, Servili M, Taticchi A, Biggeri A, Dolara P, Giovannelli L. Long-term dietary extra-virgin olive oil rich in polyphenols reverses age-related dysfunctions in motor coordination and contextual memory in mice: Role of oxidative stress. Rejuvenation Res 2012;15:601–612.
11. Cicerale S, Lucas LJ, Keast RS. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Curr Opin Biotechnol 2012;23:129–135.
12. Soler A, Romero MP, Macia A, Saha S, Furniss CSM, Kroon PA, Motilva MJ. Digestion stability and evaulation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. Food Chem 2001;119:703–714.
13. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 2009;7:65–74.
14. Gimeno E, de la Torre-Carbot K, Lamuela-Raventos RM, Castellote AI, Fito M, de la Torre R, Covas MI, Lopez-Sabater MC. Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. Br J Nutr 2007;98: 1243–1250.
15. De la Torre-Carbot K, Chavez-Servin JL, Jauregui O, Castellote AI, Lamuela-Raventos RM, Fito M, Covas MI, Munoz-Aguayo D, Lopez-Sabater MC. Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: Determination by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry. Anal Chim Acta 2007;583:402–410.
16. Gonzalez-Santiago M, Fonolla J, Lopez-Huertas E. Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. Pharmacol Res 2010;61:364–370.
17. Ruano J, Lopez-Miranda J, Fuentes F, Moreno JA, Bellido C, Perez-Martinez P, Lozano A, Gomez P, Jimenez Y, Perez Jimenez F. Phenolic content of virgin olive oil improbe ischemic reactive hyperemia in hypercholesterolemic patients. J Am Coll Cardiol 2005;46:1864–1868.
18. Biswas SK, Newby DE, Rahman I, Megson IL. Depressed glutathione synthesis precedes oxidative stress and atherogenesis in Apo-E(S/S) mice. Biochem Biophys Res Commun 2005;338:1368–1373.
19. Corona G, Spencer J, Dessi M. Extra virgin olive oil phenolics: Absorption, metabolism, and biological activities in the GI tract. Toxicol Ind Health 2009; 25:285–293.
20. Konstantinidou V, Covas M-I, Munoz-Aguayo D, Khymenets O, De la Torre R, Saez G, Tormos MdC, Toledo E,

Marti A, Ruiz-Gutierrez V, Ruiz Mendez MV, Fito M. In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: A randomized controlled trial. FASEB J 2010;24:2546–2557.
21. Khymenets O, Fito M, Covas MI, Farre M, Pujadas MA, Munoz D, Konstantinidou V, De la Torre R. Mononuclear cell transcriptome response after sustained virgin olive oil consumption in humans: An exploratory nutrigenomics study. OMICS 2009;13:7–19.
22. Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q, Lee CH, Smith AB, Breslin PAS. Phytochemistry: Ibuprofen-like activity in extra-virgin olive oil. Nature 2005; 437:45–46.
23. Zhang X, Cao J, Zhong L. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. Naunyn Schmiedebergs Arch Pharmacol. 2009;379:581–586.
24. Iacono A, Gomez R, Sperry J, Conde J, Bianco G, Meli R, Gomez-Reino JJ, Smith AB, Gualillo O. Effect of oleocanthal and its derivatives on inflammatory response induced by lipopolysaccharide in a murine chondrocyte cell line. Arthritis Rheum 2010;62:1675–1682.
25. Elnagar AY, Sylvester PW, El Sayed KA. (S)-Oleocanthal as a c-Met inhibitor for the control of metastatic breast and prostate cancers. Planta Med 2011;77:1013–1019.
26. Khanal P, Oh WK, Yun HJ, Namgoong GM, Ahn SG, Kwon SM, Choi HK, Choi HS. p-HPEA-EDA, a phenolic compound of virgin olive oil, activates AMP-activated protein kinase to inhibit carcinogenesis. Carcinogenesis 2011;32:545–553.
27. Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ. Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. Br J Nutr. 2008;100:542–551.
28. Nicola Luigi Bragazzi. Situating nutri-ethics at the junction of nutrigenomics and nutriproteomics in postgenomics medicine. Curr Pharmacogenomics Person Med. 2013;11: 162–166.
29. Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, Spencer JP. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. Biochem Biophys Res Commun 2007;362:606–611.
30. Di Bona D, Giulia Accardi, Virruso C, Candore G, Caruso C. Association between genetic variations in the insulin/insulin-like growth factor (IGF-1) signalling pathway and longevity: A systematic review and meta-analysis. Current vascular Pharmacol 2013; Dec 18. [Epub ahead of print]

Address correspondence to:
Calogero Caruso
Immunosenescence Unit
Department of Pathobiology
and Medical and Forensic Biotechnologies
University of Palermo
Corso Tukory 211
90134 Palermo
Italy

E-mail: calogero.caruso@unipa.it

Received: November 12, 2013
Accepted: November 12, 2013

CHAPTER 2

FASTING-MIMICKING DIET (FMD): MANIPULATING THE GUT MICROBIOTA IN MICE

2.1 INTRODUCTION

How discussed in the Chapter 1, in rodents, both dietary restriction (DR) and mutations in nutrient signaling pathways can extend longevity by 30-50% and lower the incidence of age-related loss of function and disease, including tumors and neurodegeneration. DR also increases "healthspan" and protects against diabetes, cancer, and cardiovascular disease in rhesus monkeys, and in humans it causes changes that protect against these age-related pathologies (*Omodei et al 2011*). In particular, fasting, the most extreme form of DR, can be applied in a chronic manner, as intermittent fasting (IF) or periodically as cycles of prolonged fasting (PF) lasting 2 or more days. PF are emerging as a highly effective strategy to protect normal cells and organs from a variety of toxins and toxic conditions (*Raffaghello et al., 2008; Verweij et al., 2011*) while increasing the death of many cancer cell types (*Lee et al., 2012; Shi et al., 2012*). PF causes a decrease in blood glucose, insulin, and insulin-like growth factor 1 (IGF-1) (*Lee et al., 2010*). Recently, a study has shown that PF causes a major reduction in the levels of white blood cells followed by stem

cell-based immune system regeneration upon refeeding (*Cheng et al., 2014*). However, prolonged water-only fasting is difficult for the great majority of the population, and its extreme nature could cause adverse effects, which include the exacerbation of previous malnourishments and dysfunctions, particularly in old and frail subjects. These concerns point to the need for dietary interventions that induce PF-like effects while minimizing the risk of adverse effects and the burden of complete food restriction. Brandhorst et al. have identified a plant-based diet that mimics the effects of fasting (fasting mimicking diet, FMD) on markers associated with the stress resistance caused by PF, including low levels of glucose and IGF-1 and high levels of ketone bodies and IGFBP-1 (*Longo and Mattson, 2014*). They discovered that cycles of the FMD lasting 4 days followed by a standard ad libitum diet promotes healthspan in mice. In fact, at the end of the FMD and before re-feeding, blood glucose levels were 40% lower than those in the control diet group. Throughout the study, glucose returned to normal levels within 7 days of re-feeding. Ketone bodies increased 9-fold by the end of the FMD but returned to normal levels after re-feeding. Serum insulin levels were reduced 10-fold after 4 days of the FMD and returned to baseline levels after re-feeding. IGF-1 was reduced by 45% by the end of the FMD period but returned to normal levels, even after multiple FMD cycles. IGFBP-1, which inhibits IGF-1, increased 8-fold by the end of the FMD regimen, but its concentration returned to levels similar to those for ad libitum mice within 1 week of re-feeding. These results indicate that FMD cycles can have profound effects on visceral fat, glucose, and IGF-1 levels, but in mice the latter changes are reversed by the return to the ad

libitum diet (*Brandhorst et al., 2015*). The age-associated decline in hematopoiesis causes a diminished or altered production of adaptive immune cells, a phenomenon known as ‘‘immunosenescence,’’ manifested as a shift in the lymphoid-to-myeloid ratio and elevated incidence of anemia and myeloid malignancies (*Muller-Sieburg et al., 2004; Shaw et al., 2010*). 4 months of FMD cycles resulted in an increase in red blood cell count and hemoglobin levels compared to baseline. Complete blood counts indicated that the FMD causes a rejuvenation of the blood profile and a reversal of the age-dependent decline in the lymphoid-to-myeloid ratio (L/M), as well as of the age-dependent decline in platelets, and hemoglobin. These results indicate that chronic use of the FMD promotes immune system regeneration and rejuvenation, in agreement with previous results on the effect of fasting on lymphocyte number (*Cheng et al., 2014*). Cycles of FMD are effective in promoting increases in hematopoietic and mesenchymal stem and progenitor cells, which are likely to contribute to the regeneration of various cell types/systems. Moreover, FMD reduces cancer incidence in C57BL/6J mice, promotes hippocampal neurogenesis, improves cognitive performance and increases Median lifespan (11% extention). The FMD showed an 18% extension effect at the 75% survival point, but only a 7.6% extension effect on the 25% survival point and no effect on maximum lifespan. In humans three monthly cycles of a 5-days FMD reduced multiple risk factors of ageing. In fact, they conducted a pilot clinical trial in healthy adult to evaluate the impact of the FMD in humans. The trial included a set of 19 participants who successfully completed 3 FMD cycles and 19 partecipants who were randomized to continue on their normal diet

and serve as control. The FMD showed to have a beneficial effect in the glucose, IGF-1, IGFBP-1, ketone bodies, C reactive protein levels and in the percentages of body weight, trunk fat and lean body mass. Moreover, the percentage of MSPC in the peripheral blood mono-nucleated cell population showed a trend ($p = 0.1$) to increase from 0.15 ± 0.1 at baseline to 1.06 ± 0.6 at the end of FMD, with a subsequent return to baseline levels after re-feeding (*Brandhorst et al., 2015*).

2.2 Factors influencing the gut microbiota

The human gut microbiota plays a key role in numerous metabolic, physiological, nutritional and immunological processes (*O'Hara AM and Shanan, 2006*) and discomposure or disturbance in the functions of the microbiome are linked to inflammatory and metabolic disorders affecting the human health (*O'Toole PW and Claesson*, *2010*). For many years the microbiota has been investigate by using anaerobic culture techniques which have provided important information concerning the bacteria populations colonizing the human gut (*Moore and Moore, 1995*). However, the classical culture methods are labor-intensive and time consuming, in addition, the classification and identifications based on phenotypic traits do not always allow clear results. Molecular techniques based on 16S rRNA sequences permitted the detection and identification of bacterial species that are difficult to culture, providing power tools to study the microbiome, "the organ containing 150-fold more genes than the human genome". (*Clarridge J. 2004; Vanughan et al., 2000; O'Sullivan et al., 2000*; *Dethlefsen et al., 2006).* The microbial content changes along the length of the gastrointestinal tract (GIT), varying from a attenuated diversity and a low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine (*Tiihonen et al., 2010*). The human intestine is populated by 10^{13} to 10^{14} micro-organisms, the vast majority of which belong either to the phylum Firmicutes or to the phylum Bacteroides including *Lactobacillus, Clostridium, Ruminococcus, Enterococcus, Bacteroides and Prevotella* which constitute over 90% of the known philogenetic

categories found in the human intestine (*Qin et al., 2010*; *Eckburg et al., 2005; Wang et al., 2003*). Since the gestation mother, several factors influence the microbiome composition because the flora originates in the canal birth where bacterial populations, inhabiting the maternal vaginal, colonize the intestine. Initially, facultative anaerobes such as *Enterobacteriaceae*, streptococci and staphylococci dominate (*Marque et al.,, 2010*). For infants delivered by cesarean section, the environment such as nursing tools and the air, is the dominant source of colonizing microorganisms impacting on the intestinal flora with a lower bacterial counts and less diversity than in infants born vaginally (*Grolund, 1999*). Another factor influencing the composition of the microbiota in the early infancy is the newborn feeding. The microbiota of formula-fed infants includes *Enterobacteriaceae, Streptococcus, Bacteroides, Clostridium* and *Bifidobacterium*, against the microbiota of breast-fed infants is dominated by *Bifidobacterium* and *Ruminococcus* with ratios of colonization by *Escherichia coli, Clostridium difficile, Bacteroides Fragilis group* and Lactobacilli being significantly lower than those found in the formula-fed infants (*Penders J, et al 2006; Favier et al, 2002*). However, the intestinal microbiota in early infancy is characterized by instability and its composition changes further with the introduction of solid food, becoming more stable, similar to the adult, after 2-3 years of age (*Yatzunenko T et al, 2012; Favier C et al, 2002*). Although the intestinal microbiota is relatively stable throughout adulthood, age – related changes in the gastrointestinal tract (GIT), as well as changes in diet and host immunity reactivity, inevitably affect bacteria population composition (*Delgado S et al, 2006*). The normal intestine

microbiota is important for the maintenance of the host health, providing energy in the form of short-chain fatty acid (SCFAs) (*Cummings et al., 1997*), nutrients such as vitamins K and B12 (*Deguchi et al 1985*) and protection against invading microorganisms by exerting colonization resistance (*Van der Waaij et al 1971; Rolfe 1997*). Physiological changes, as decreased intestinal motility and slow transit times, occur in the GI tract of elderly people, resulting in a reduction of the faecal weight and reduced excretion of bacterial matter (*Woodmansey et al, 2004; Stephen et al, 1987*). The prolonged retention time leads to an increase in bacterial protein fermentation and consequently putrefactive processes in the gut with a greater susceptibility to disease (*Macfarlane et al, 1989*). Changes with age in specific bacterial genera and species have been identified, with a considerable inter-individual variation that continues into old age (*Claesson MJ et al, 2011*). Many studies have shown a decline, with the increased age, in counts of *Bacteroides* and *Bifidobacteria*, either in the total numbers and in the species diversity, that is amplified consequently to the antibiotic therapy (*Hopkins and Macfarlane, 2002; Woodmansey, 2004; Bartosch et al. 2004*). Bacteroides, being able to utilize a wide variety of different carbon sources, are responsible for the majority of polysaccharide digestion in the colon (*Salyers 1984; Macfarlane and Gibson, 1991*), accordingly, the decreased Bacteroides species level correlates with a reduction in amylolytic activity observed in healthy elderly population (*Woodmansey E.J et al. 2004*). In concomitance to the shifts observed in the genus Bacteroides, the decreasing of the beneficial bifidobacteria levels is one of the most notable changes in the elderly

gut. A wide number of bifidobacterial species are observed in infants and young adults, however, only few species are found in the elderly population, in particular *Bifidobacterium adolescentis* and *Bifidobacterium longum* (*Gavini et al., 2001*). Moreover, evidences support an increase in the number of facultative anaerobes, fusobacteria, clostridia and eubacteria, with a rise in the proteolityc activity, particularly following antibiotic treatment (*Woodmansey; 2007*). These changes in the microbial composition can be linked to the increased serum antibodies levels to commensal microorganisms, such as *Escherichia coli* and *Enterococcus faecalis*, in a healthy adult population (*Percival et al. 1996*). A study conducted by the ELDERMET consortium reports that the microbial population of elderly Irish subjects is dominated by Bacteroides against the microbiota of younger subjects dominated by Firmicutes (*Claesson et al. 2011*). However, significant differences have not been found among the Firmicutes:Bacteroides ratio of Italians centenarians, elderly and young adults (Biagi et al. 2010). These conflicting results can be attributed to the country- related variation in the composition of the microbiota as well as the different residence locations, which reflect two radically different diets (*Biagi et al. 2012; Claesson et al. 2011*).

2.3 Macrobiota: Diet and genetic

Mammals exhibit marked inter-individual variations in their gut microbioma and the host genotype has a measurable impact on gut microbiota composition in both mice (*Benson et al., 2010*) and humans (*Goodrich et al., 2014*); but comparisons of human twins at various ages, from infants to adults, have failed to detect significantly more similar microbial communities in monozygotic versus dizygotic pairs, suggesting that environmental factors predominate over host genetics in shaping microbial ecology. (*Turnbaugh et al., 2009; Yatsunenko et al., 2012*).

Diet is known to modulate the composition of the microbiota in humans and mice. David et al. show that the short-term consumption of diets composed entirely of animal or plant products alters microbial community structure and overwhelms inter-individual differences in microbial gene expression. (*David et al., 2014*)

Time series analyses of inbred mice have shown that the consumption of a high-fat, high sugar (HFHS) diet alters the gut microbiota in a single day (*Turnbaugh et al., 2009; Zhang et al., 2012*) and the microbial response to the consumption of the HFHS diet overshadows preexisting genetic association. Male C57BL/6J wild type mice, and animals homozygous for mutations in four genes, that have been previously shown to impact the gut microbiota, were fed a low- fat, high-plant-polysaccharide (LFPP) diet for 4 weeks at which point they were switched to the HFHS for 1 week. Analysis of microbial community structure over time revealed that all genotypes responded within 2 days. The microbial response seems to be proportional to the

degree of dietary perturbation when the mice are fed with different percentages HFHS by weight. Moreover, the abundance of taxa depends on numbers of prior shifts diet. Analysis of the consecutive dietary shifts in outbread mice suggested that both community membership and structure were markedly altered after 3-7 days of HFHS diet consumption in naive mice, whereas the gut microbiota of mice previously exposed to the HFHS diet took 1-2 weeks to respond (*Carmody et al., 2015*). Changes in the composition of the gut microbioma in response to dietary intake occur most because different bacterial species are better equipped to utilize different substrates (*Scott KP et al*., *2008*). Generally bacteria prefer carbohydrates as primary energy sources if they are available (*Apajalahti et al*., *2005*). Analysis of fecal short-chain fatty acid (SCFAs) and bacteria clusters suggests that macronutrient shifts on plant-based diet and animal-based diet also alter microbial metabolic activity. Correlating subject's SCFA concentration with bacterial clusters, it was observed significant positive correlation between clusters composed of putrefactive microbes and SCFAs that are the end products of the amino acid fermentation (*Alistipes putrenidis and Bacteroides*) and clusters comprised of saccharolytic microbes, for example, *Rosesburia, E rectale and F. prausnitzii,* and the products of carbohydrate fermentation. (*David et al., 2014*). Saccharolytic and proteolytic bacterial fermentation take place mainly in the proximal and distal colon, respectively, and result in the production of SCFA and in addition to ammonia, amines, phenols, thiols and indoles in theamino acid fermentation (*Guarner and Malagelada, 2005*).

However, the plant-and animal-based diet reflect transcriptional

response that are consistent with differences in gene transcription between the gut microbiomes of herbivorous and carnivourous mammals. For example the animal- based diet was associated with significant increase in bacterial gene expression among glutamine amido transferases (Vitamin B6), methyltransferase (polycyclic aromatic hydrocarbon degradation) and beta-lactamase. (*David et al. 2014).*

SCFAs produced from microbial fermentation affect proliferation, differentiation and modulation of gene expression in mammalian colonic epithelial cells. These effects have been attributed to butyrate acting as a potent histone deacetilase inhibitor and, as such, it may regulate 2% of the mammalian transcriptome. SCFAs can regulate gene expression by binding to the G-protein-coupled receptors (GPCRs), GPR41 and GPR43. SCFAs suppress inflammation through GPR43 signaling in immune cells and modulate secretion of the hormone GLP1, which improves insulin secretion and has antidiabetic effects. (*Tremaroli and Backhed, 2012*).

2.4 AIM 2 OF THE THESIS

Reduced food intake, avoiding malnutrition, can ameliorate aging and aging-associated diseases in invertebrate model organisms, rodents, primates, and humans. These effects seem to be dependent in part on the reduction of the nutrient signaling pathways associated with decreased circulating IGF-1 and glucose levels Modulation of the gut microbiota through the diet can also be important to promote healthy effects in the organisms (*Fontana and Partridge, 2015*). The human intestinal microbiota plays a fundamental role in numerous metabolic, physiological, nutritional and immunological processes and manipulating the gut microbiota may result in modification of functionality of an aged immune system (*O'Hara and Shanan, 2006*). Diet is known to modulate the composition of the microbiota in humans and mice. Long-term dietary intake is known to influence the structure and the activity of the trillions of microorganisms in the human gut, but it remains unclear how rapidly and reproducibly the human gut microbiome responds to short-term macronutrient change. David et al. show that the short-term consumption of diets composed entirely of animal or plant products alters microbial community structure and overwhelms inter-individual differences in microbial gene expression (*David et al 2014*).

During the last year and half of my research activity, carried out at the "Longevity Institute" of the "University of Southern California", I examined the effect of the dietary intervention on the gut micro biome, with the main aim to define a common bacteria profile correlated to the short–time starvation (STS) and fasting- mimicking

diet (FMD). In the first phase of the project, I designed a study to determine the time points, relating to the collection of fecal samples, and in order to standardize a suitable method that allowed me to quantify the bacterial species of interest. Bacterial groups and species-specific were quantified in each group (see details below) using a quantitative real- time PCR (qPCR) based on previously published specific primers for total bacteria (HAD primers), *Bifidobacterium group, Enterobacteriaceae, Prevotella group, Lactobacillus, Faecalibacterium prausnitzii, Eubacterium rectale and Rosesburia intestinalis*, designed on the gene coding for 16S ribosomal subunit.

After the study described above, I examined the effect of short-term starvation (STS) and fasting- mimicking diet (FMD) in the gut microbiota of a "nude mouse" model to test the effects of the diets in the gut microbiota in an immune-deficient condition. At the end, I tested the FMD effect in wild-type, 18 months- old, mice.

2.5 RESULTS

To define a bacterial profile correlated to the Short time starvation (STS) and fasting-mimicking diet (FMD), I considered two different conditions that differ by age and chow food in which the STS and FMD have previously shown to have an effect in the mice. In the first experiment, 3 groups of C57BL/6J wild-type female mice, 12-weeks old, were fed: Group1: Mice undergo with regular food ad lib (control diet); Group2: Mice undergo cycles of 4-days FMD; Group3: Mice undergo cycles of 48h short-time starvation (STS).

The fecal samples have been collected from the cages of each group of mice the last day of the 2° cycle FMD/STS and at the same time point of the control diet (ad lib). (See table 1)

Table 1: Real- time PCR quantization of 16S rRNA gene in feces of mice 12- week old

3mo, female	Control			Last day FMD				Last day STS			
	Mean (Ct)	SD (Ct)	N (ng/ul)	Mean (Ct)	SD (Ct)	N (ng/ul)	N-Fold	Mean (Ct)	SD (Ct)	N (ng/ul)	N-Fold
*HAD (*total bacteria*)*	29,74	0,80	0,70	17,51	0,30	1,68	2,39	23,75	0,01	0,33	0,47
Bifidobacterium group	28,81	0,14	2,13	27,06	0,00	31,75	6,23	30,10	0,05	0,35	0,35
Enterobacteriaceae group	33,09	0,06	1,36	28,10	0,04	572,60	176,60	29,47	0,17	110,74	173,36
Lactobacillus group	21,15	0,07	1,32	22,62	0,01	0,52	0,17	23,44	0,03	0,31	0,50
Prevotella group	31,43	0,16	1,19	32,56	0,09	0,63	0,22	31,93	0,00	0,90	1,60
Faecalibacterium prausnitzii	28,39	0,30	0,07	29,84	0,11	0,02	0,14	29,94	0,04	0,02	0,61
Eubacterium rectale	28,85	0,14	2,97	28,11	0,06	0,71	0,10	27,17	0,42	1,31	0,94
Rosesburia intestinale	20,22	0,14	1,49	20,15	0,00	1,55	0,43	20,54	0,08	1,27	1,81

a.Mean: indicates the mean value of the two Ct values
b.SD: indicates the standard deviation of the Ct values
c.N(ng/ul): indicates the value obtained through standard curves created with a "control sample"of known concentration
d.N-fold: indicates the value N divided the total bacteria value respect to the control

Of the groups and species analyzed, *Enterobacteriaceae* increases 176,60-fold respect to the control group the last day of the 2° cycle of FMD and 173,36- fold the last day of the 2° cycle of STS.

Bifidobacterium group seems increased of around 6-fold respect to the control after the 2° cycle of the FMD, the others species seem decreased or not change after dietary intervention.

In the second experiment 4 groups of C57BL/6J wild-type female mice, 16-months old:

Group1: mice undergo High Fat Diet (HFD); Group2: Mice fed with HFD and undergo cycles of FMD (5 days + 2 days of standard diet) and return to dietary regimen HFD for 3 weeks; Group 3: Mice fed with Standard diet.

The fecal samples have been collected the day before the 9° FMD cycle (Before FMD), after 5 days FMD (+ 2 days Standard diet) and at the same time points of Standard and High fat diet. (See table 2).

Table 2: Real- time PCR quantization of 16S rRNA gene in feces of mice 16- months old

High fat diet experiment	HFD			Before 9° cycle FMD				After 9° cycle FMD				Standard diet		
	Mean (Ct)	SD (Ct)	N (ng/ul)	Mean (Ct)	SD (Ct)	N (ng/ul)	N-Fold	Mean (Ct)	SD (Ct)	N (ng/ul)	N-Fold	Mean (Ct)	SD (Ct)	N (ng/ul)
*HAD (*total bacteria*)*	19,50	0,21	4,40	20,53	0,17	2,89	0,66	24,42	0,13	0,59	0,20	30,60	0,74	0,05
Bifidobacterium group	28,05	0,33	2,51	29,56	0,03	0,14	0,08	29,38	0,08	0,20	7,07	30,49	0,20	0,02
Enterobacteriaceae group	36,13	0,11	0,02	35,61	0,00	0,11	9,40	36,96	0,21	0,02	0,97	35,69	0,30	0,10
Lactobacillus group	21,49	0,14	19,17	22,19	0,14	13,00	1,03	34,50	0,12	0,02	0,01	32,96	0,07	0,05
Prevotella group	32,89	0,16	0,04	33,57	1,32	0,01	0,26	37,42	0,29	0,00	0,11	38,05	0,01	0,00
Faecalibacterium prausnitzii	33,24	0,53	0,01	33,94	0,14	0,00	0,61	35,99	0,08	0,00	0,73	35,52	0,07	0,00
Eubacterium rectale	32,99	0,01	0,05	32,19	0,16	0,07	2,02	37,41	0,51	0,00	0,14	38,64	0,00	0,00
Rosesburia intestinale	22,47	0,12	0,61	21,42	0,03	1,04	2,60	25,58	0,20	0,12	0,56	28,13	0,04	0,03

a.Mean: indicates the mean value of the two Ct values

b.SD: indicates the standard deviation of the Ct values

c.N(ng/ul): indicates the value obtained through standard curves created with a "control sample"of known concentration

d.N-fold: indicates the value N divided the total bacteria (relative abundance) and respect to the Group1 HFD

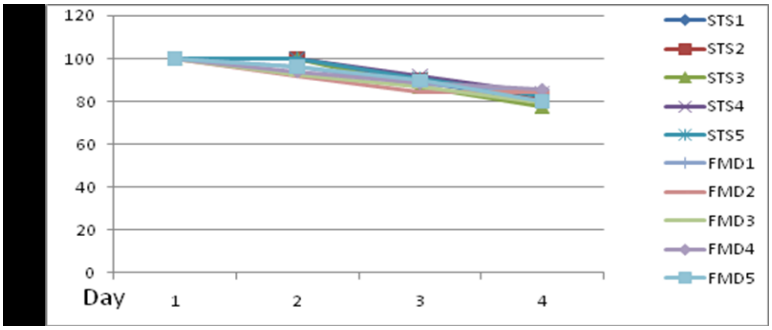
e.N-fold: indicates the value N divided the total bacteria (relative abundance) and respect to the Group2 Before FMD

The total bacteria related to the HAD primers seem decreased 0,20-fold after FMD respect to the Group 2 Before FMD; the amount of *Lactobacillus*, equal in the Group 1 and in Group 2 Before FMD, drops down the last day of FMD (0,01-fold). The abundance of *Bifidobacterium* increased 7,07-fold at the end of the FMD respect to the Group 2 Before FMD and the abundance of *Enterobacteriaceae* was higher 9,40-fold in the Group 2 respect to the Group 1. The amounts of all species and groups were considerably lower in the mice undergo the Standard Diet (Group 3).

After evaluating the effectiveness of the method and considering some adjustments for the sample collection, I examined the effect of the STS and FMD in the gut microbiota of a "nude mouse" model. Therefore, I designed an experiment involving 20 mice athymic, Balb/C: 5 mice undergo 3-days FMD followed by 7 days of a regular dietary regimen (re-feeding, RF); 5 mice undergo 48hr STS followed by 7 days of re-feeding (RF); 5 mice undergo 3-days FMD and 5 mice undergo 48hr STS.

The mice treated with STS and FMD have lost ~ 20% weight during 2- days STS and 3-days FMD and reached the initial weight after 7 days re-feeding. The body's weight has been measured every day during the diets and after 7 days re-feeding. (See figures below).

A)



B)

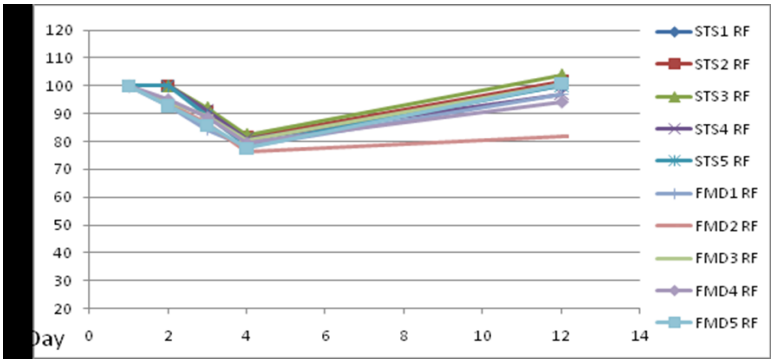


Figure 1.A.Weight loss percentage in mice undergo STS from day2 to the day4 and in mice undergo FMD. B. Weight loss during the diets and regained after 7 days re-feeding (RF)

The bacterial species quantified in this study are: Total bacteria, *Lactobacillus, Bifidobacterium, Enterobacteriaceae* and *Faecalibacterium prausnitzii*. The fecal samples have been collected at the Baseline, before the dietary intervention and at the end of the treatments (STS, FMD, STS-RF, FMD –RF). (See Figures below). The samples have been analyzed contemporary for every fecal specie investigated.

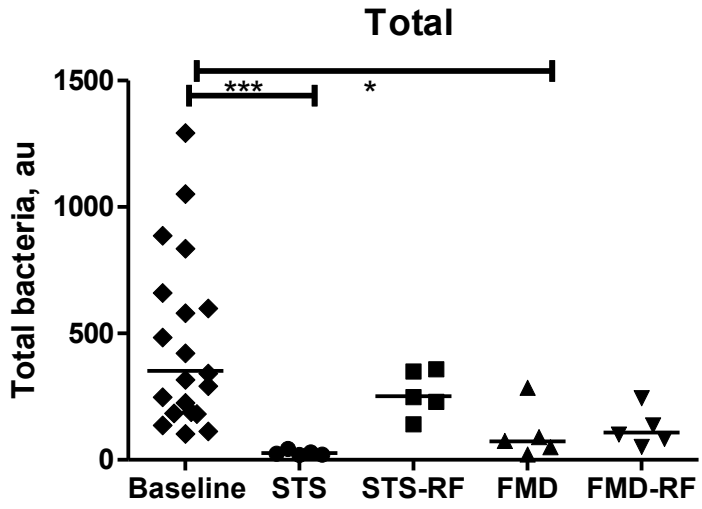


Figure 2. au: indicates arbitrary unit; amount obtained in 0,1 gr of stool. The amount was calculated for each mouse, and the geometric mean was determined for each group.
A non parametric Mann-Whitney U test was used for the comparison of the groups.
Bars represent the geometric mean. *, significant difference($p<0.05$) between groups Baseline and FMD;***, significant difference between Baseline and STS.
Linear scale in the Y axis.

The total bacteria amount decreased after treatment 48hr STS (significant reduction; p=0.0008) and tends to reach the initial level after 1 week re-feeding (STS-RF); also the treatment FMD acts by decreasing the number of total bacteria (significant reduction; p=0.048), which does not reach the initial levels after 7 days re-feeding (FMD- RF).

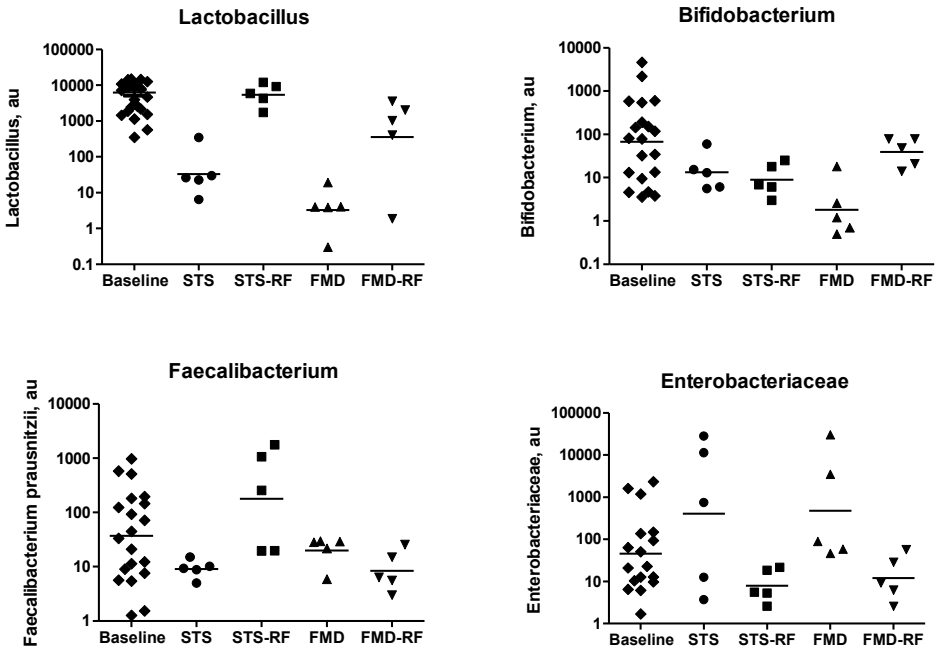


Figure 3.au: indicates arbitrary unit; amount obtained in 0,1 gr of stool. The amount was calculated for each mouse, and the geometric mean was determined for each group.
Bars represent the geometric mean. Log-scale in the Y axis.

The abundance of the specific rRNA genes were calculated for each mouse, and the mean of each mice group was determined to assess reductions or increase in certain bacterial populations after dietary interventions. The abundance of *Lactobacillus* was significantly lower in stool samples from both groups STS (p=0.0113) and FMD (p=0.0111), compared to "Baseline" results, and reaches the initial levels in the STS-RF and FMD-RF mice.

As it regards the group *Bifidobacterium*, Fasting-mimicking diet acts more, compared to Short-time starvation, in reducing the quantity which increases considerably after re-feeding (FMD vs FMD-RF p=0.0357). It also notables that the amount of *Enterobacteriaceae*

found to be increased in the groups STS and FMD, becomes lower and more consistent after 7 days of the regular diet. The diets seem to have no significant effects on *Facaelibacterium prausnitzii*. These data show that the Short-time starvation, STS, and Fasting-mimicking diet, FMD, manipulate the gut microbiota in the "nude mice" (Figure 3).

At the end, I tested the effect of the FMD in the gut microbiota of C57BL/6J , 18- months old mice. This time, I considered 3 time points with 3 groups of 4 mice each: FMD-i : mice undergo 2 cycles 4-days FMD, FMD-i-14dRF: mice undergo 2 cycles 4 -days FMD followed by 14 days re-feeding, FMD-i-34dRF: mice undergo 2 cycles 4-days FMD followed by 34 days re-feeding. In every time point considered, I collected the fecal sample in a control group fed with an ad libitum diet to compare the results.

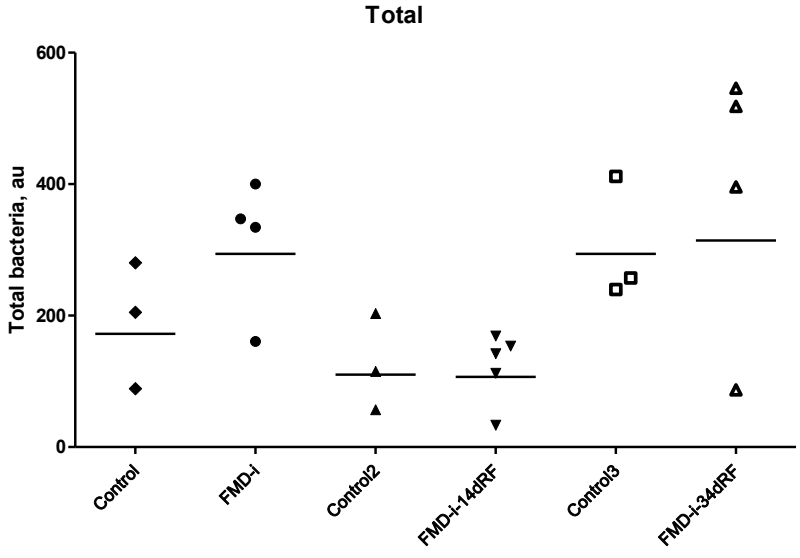


Figure 4. au: indicates arbitrary unit; amount obtained in 0,1 gr of stool.
The amount was calculated for each mouse, and the geometric mean was determined for each group.
Linear scale in the Y axis.

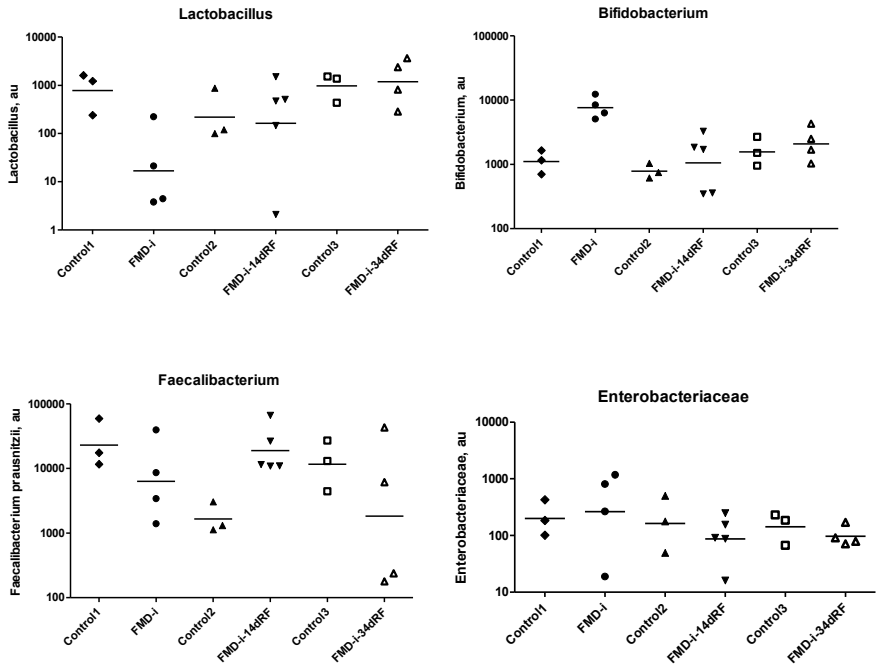


Figure 5.au: indicates arbitrary unit; amount obtained in 0,1 gr of stool. The amount was calculated for each mouse, and the geometric mean was determined for each group. Bars represent the geometric mean. Log-scale in the Y axis

Significant differences in the number of total bacteria are not found among the treatment groups (FMD-i, FMD-14dRF, FMD 34dRF) and respective controls (Control 1, Control 2, Control 3). The amount of *Lactobacillus group* is decreased at the end of the FMD-i (p=0.0391) and reaches values equal to the respective controls after 14 days and 34 days re-feeding. Interestingly, *Bifidobacterium group* levels increased after the 2° cycle of FMD-i (p=0.0152) respect to the control and returned to the initial levels after 14 days and 34 days re-feeding. The specie *Faecalibacterium prausnitzii* is more abundant in the FMD-i 14dRF respect to the Control 2 but the levels are equal at the Control 1. Changes have not involved the *Enterobacteriaceae group.*

2.6 DISCUSSION

The gut microbiota plays the principal role to maintaining human health (*Chung and Kasper, 2010*) and its homeostasis is inexorably altered by age-related physiological changes in the gastrointestinal tract induced not only by the aging process itself, but also by modifications in lifestyle, nutritional behavior, and functional reduction of the host immune system (*Biagi et al., 2011*). In turn, the age-related gut microbiota alterations influence the aging process in the host, principally, immunosenescence, age-dependent inflammatory status and its complications from metabolic syndrome – diabetes, cardiovascular diseases (CDs), and cancer – and cognitive decline – dementia and Alzheimer's disease (AD) (*Candore et al. 2008*).

Diet is known to modulate the composition of the microbiota in humans and mice. Short-term consumption of diets composed entirely of animal or plant products alters microbial community structure (*David et al., 2014*) and alterations in the gut microbiome may contribute to the improvement in health from DR and time-restricted feeding. Thus, the aim of my study was to investigate the effects of STS and FMD in the gut microbiota of mice to assess whether some beneficial effects of these diets on the health can be attributed to their ability to manipulating the microbiota. The study on the nude mouse model has permitted to verify the effects in an immune-deficient condition, considering a contest in which the immune system is compromised. Recent studies show that the gut microbiota can influence immunosenescence or an inflammatory status and viceversa a different immune system reactivity, or an host genetic variation in

immunity- related pathways, can modulate the microbiota composition (*Salzman et al., 2010; Blekhman et al., 2015, Lupp et al., 2007).* From the data obtained in the "nude mice experiments", it was observed that both the dietary interventions, STS and FMD; act in similar manner. In particular, STS has reduced the total bacteria number that increased after 1 week refeeding, also FMD has reduced the amount of the total bacteria which, in contrast to the STS-RF mice, doesn't reach the initial levels after 7 days re-feeding. Significant changes in the amount of the total bacteria are not observable in the wild type old mice; probably the immune system contributes to maintain stable the number of the total bacteria in the gut.

Interestingly, the abundance of *Lactobacillus group was* significantly lower at the end of STS and FMD respect to the "Baseline" levels and reaches the initial levels after 1 week re-feeding in the nude mice, also in the wild-type mice the FMD-i reduces the *Lactobacillus* levels which increase again after 14 days and 34 days re-feeding. These results are consistent with a recent study in which time-restricted feeding during 8 hr of the dark phase decreased representation of the *Lactobacillus species* in mice fed with high-fat diet (*Zarrinpar et al., 2014*), similarly to the results shown in Table 2 where, in mice undergo high fat diet, the amount of *Lactobacillus* drops down after FMD treatment. Recent studies have shown an association between several *Lactobacillus species* and obesity (*Joyce et al., 2014, Million et al 2012, Million et al 2013*); in particular a decrease in *Lactobacillus species* protects against metabolic disorders associated to obesity, perhaps by altering bile acids in the lumen (*Li et al., 2013*).

On the light of these observations, the FMD could have a protective effect against obesity and metabolic disorders.

As evidenced by results, cycles of FMD increase the abundance of the *Bifidobacterium group* at the end of the diet, except in the nude mice where the *Bifidobacterium* increases after 1 week re-feeding. The *Bifidobacterium* species have been shown to lower intestinal LPS levels and to improve mucosal barrier function in Balb/c mice *(Griffiths et al., 2004)*. Moreover, one of the most notable change in the elderly gut is the decreasing of the beneficial bifidobacteria levels. A wide number of bifidobacterial species are observed in infants and young adults, however, only few species are found in the elderly population, in particular *Bifidobacterium adolescentis* and *Bifidobacterium longum*. (*Gavini et al., 2001*). The FMD could improve health effects through increasing the abundance of *Bifidobacterium* in elderly. A recent study showed that commensal *Bifidobacterium* alone, transplanted in Balb/c mice, improved tumor control to the same degree as programmed cell death protein 1 ligand 1 (PD-L1)-specific antibody therapy, and combination treatment nearly abolished melanoma tumor outgrowth (*Silvan et al., 2015*).

In the nude mice, higher values of *Enterobacteriaceae* are observable at the end of STS and FMD, the values become lower and more consistent after re-feeding. We did not observe the same in the wild-type mice, in which the levels are maintained lower in all of the conditions. Probably the immune system still contributes to restrain their number.

The amount of *Faecalibacterium prausnitzii* did not differ between the mice groups analyzed, except in the STS-RF group of nude mice,

in which the number is higher respect to the baseline, also in the wild-type mice the number of *Faecalibacterium prausnitzii* is higher in the FMD-i 14dRF respect to the relative control (Control 2) but did not differ respect the other controls (Control 1 and Control 3). *Faecalibacterium prausnitzii*, saccharolytic bacteria positively correlated at the carbohydrate fermentation, is one of the dominant butyrate-producing bacteria detected in human feces and plays a beneficial role in the intestinal mucosa through the multiple effects of butyrate as the preferred energy source for the colonocytes and it is also considered to have additional anti-inflammatory properties that are suggested by cellular studies and experimental colitis models in mice (*Martin et al., 2015*)

These data show that the FMD manipulates the gut microbiota in the mice and this study provide a basis from which to consider the functional consequences of the gut microbiota modified by the diet, which could contribute to the beneficial effects, previously described, of the fasting and fasting mimicking diet, especially in the metabolism and immunity. Experimental fecal transplantations in mice of microbiota associated to the FMD could be revealing of their causal role. It will be important to determine if similar shifts in the bacterial populations occur in the context of humans undergoing analogous "yo-yo" diets, reflecting alternating periods of increased and decreased caloric intake *(Atkinson et al. 1994*). It is interesting to consider whether or not the observed microbial plasticity may be a selective trait that helped our ancestors maintain energy balance given a volatile diet that was dependent on season and foraging success (*Hawkes et al., 1991*). This concept is concord with the view that the

effects of the CR on aging and diseases are responses evolved in mammals to adapt to periods of limited availability of food (*Fontana and Klein, 2007; Fontana et al., 2010; Masoro, 2005; Weindruch and Walford, 1988*).

This present thesis strengthen the suggestion of the role played by fasting and "fasting mimicking diet" as an effective intervention to promote longevity and health span.

2.7 MATERIALS AND METHODS

DNA extraction and Real-time PCR

The fecal samples have been collected, from the cages for the experiments 1 and 2 (Table1 and Table 2), from the distal colon for the other experiments, and storage at -80°C until DNA extraction. The bacterial DNA was extracted from each fecal sample (0.1 gr) according to the MOBIO kit instructions and kept at -20°C until use. The DNA was normalized to a concentration of 1.0 ng/ul, measured by Nanodrop for subsequent real-time qPCR assay. The main bacterial groups of the intestinal microbiota were quantified by real- time qPCR using specific primers, previously published (*Fujimoto et al., 2013; Langendijk et al., 1995; Bartosch et al., 2004; Cano et al., 2010; Walter et al., 2000*) (Table 3). A Thermal Cycler CFX96 Touch Bio-rad, associated with a Software (Bio-rad CFX manager 3.1 version) was used for the real-time PCR. Each reaction was done in duplicate in a volume of 25 ul with 96 –well optical-grade PCR plates (Bio-rad). Amplification were done with the following temperature profiles: one cycle 95°C 5 min, 39 cycles of denaturation at 95°C (15 sec). Standard curves of each bacterial group were generated from serial dilutions using DNA of known concentration extracted from a "control sample", to compare the Ct values obtained in every qPCR reaction respect to it. Melt curve analyses were made by heating the PCR mixtures from 55 C° to 95°C with simultaneous measurements of the SYBR Green signal intensities.

Mice Diets

The FMD is a plant-based diet based on a nutritional screen that identified ingredients that allow nourishment during periods of low calorie consumption (*Brandhorst et al., 2013*). The FMD consists of two different components designated as day 1 diet and day 2–4 diet that were fed in this respective order. The day 1 diet consists of a mix of various low-calorie broth powders, a vegetable medley powder, extra virgin olive oil, and essential fatty acids; day 2–4 diet consist of low-calorie broth powders and glycerol. Both formulations were then substituted with hydrogel (Clear H2O) to achieve binding and to allow the supply of the food in the cage feeders. Day 1 diet contains 7.67 kJ/g (provided at 50% of normal daily intake; 0.46 kJ/g protein, 2.2 kJ/g carbohydrate, 5.00 kJ/g fat); the day 2–4 diet is identical on all feeding days and contains 1.48 kJ/g (provided at 10% of normal daily intake; 0.01 kJ/g protein/fat, 1.47 kJ/g carbohydrates). At the end of the diet, we supplied chow ad libitum for 10 days before starting another FMD cycle. Prior to the FMD, animals were transferred into fresh cages to avoid feeding on residual chow and coprophagy.

Table 3 Specific- primers groups

Target bacterial Group/Species	Designation	Primers (5'-3')Sequence	Annealing Temperature
Total bacteria	HAD F HAD R	TGGCTCAGGACGAACGCTGGCGGC CCTACTGCTGCCTCCCGTAGGAGT	59°C
Bifidobacterium group	Bif164F Bif601R	GGGTGGTAATGCCGGATG TAAGCCATGGACTTTCACACC	58°C
Lactobacillus group	Lac F Lac R	AGCAGTAGGGAATCTTCCA ATTYCACCGCTACACATG	61°C
Enterobacteriaceae group	Entero F Entero R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	63°C
Faecalibacterium prausnitzii	Frp-2F Fprau645R	GGAGGAAGAAGGTCTTCGG AATTCCGCCTACCTCTGCACT	55°C

(*Fujimoto et al., 2013; Langendijk et al., 1995; Bartosch et al., 2004; Cano et al., 2010; Walter et al., 2000*)

REFERENCES

Albani D, Batelli S, Polito L, Vittori A, Pesaresi M, Gajo GB, De Angeli S, Zanardo A, Gallucci M, Forloni G. A polymorphic variant of the insulin-like growth factor 1 (IGF-1) receptor correlates with male longevity in the Italian population: a genetic study and evaluation of circulating IGF-1 from the "Treviso Longeva (TRELONG)" study. BMC Geriatr 2009;9:19.

Apfeld J, Kenyon C. Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell 1998;95:199-210.

Anselmi CV, Malovini A, Roncarati R, Novelli V, Villa F, Condorelli G, Bellazzi R, Puca AA. Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. Rejuvenation Res 2009;12:95-104.

Apajalahti J. Comparative gut microflora, metabolic challenges, and potential opportunities. J Appl Poult Res 2005;14, 444–453.

Arking DE, Krebsova A, Macek M Sr, Macek M Jr, Arking A, Mian IS, Fried L, Hamosh A, Dey S, McIntosh I, Dietz HC. Association of human aging with a functional variant of klotho. Proc Natl Acad Sci U S A 2002;99:856-61.

Arking DE, Atzmon G, Arking A, Barzilai N, Dietz HC. Association between a functional variant of the KLOTHO gene and high-density lipoprotein cholesterol, blood pressure, stroke, and longevity. Circ Res 2005;96:412-8.

Arking DE, Becker DM, Yanek LR, Fallin D, Judge DP, Moy TF, Becker LC, Dietz HC. KLOTHO allele status and the risk of early-onset occult coronary artery disease. Am J Hum Genet. 2003;72:1154-61.

Barbieri M, Boccardi V, Esposito A, Papa M, Vestini F, Rizzo MR, Paolisso G. A/ASP/VAL allele combination of IGF1R, IRS2, and UCP2 genes is associated with better metabolic profile, preserved energy expenditure parameters, and low mortality rate in longevity. Age 2012;34:235-45.

Barbieri M, Paolisso G, Kimura M, Gardner JP, Boccardi V, Papa M, Hjelmborg JV, Christensen K, Brimacombe M, Nawrot TS, Staessen JA, Pollak MN, Aviv A. Higher circulating levels of IGF-1 are associated with longer leukocyte telomere length in healthy subjects. Mech Ageing Dev 2009;130:771-6.

Bartke A. Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. Endocrinology 2005;146:3718-23.

Bartosch S, Fite A, Macfarlane GT, et al. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using realtime PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 2004; 70, 3575–3581.

Biagi E, Nylund L, Candela M, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One 2010; 5, e10667.

Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q, Lee CH, Smith AB, Breslin PAS. Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. Nature 2005;437:45-46.

Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J, Zhang M, Oh PL, Nehrenberg D, Hua K, Kachman SD, Moriyama EN, Walter J, Peterson DA, Pomp D. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci U S A. 2010;2;107(44):18933-8.

Bonafè M, Barbieri M, Marchegiani F, Olivieri F, Ragno E, Giampieri C, Mugianesi E, Centurelli M, Franceschi C, Paolisso G. Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cures for an evolutionarily conserved mechanism of life span control. J Clin Endocrinol Metab 2003;88:3299-304.

Brandhorst, S., Wei, M., Hwang, S., Morgan, T.E., and Longo, V.D.. Short-term calorie and protein restriction provide partial protection from chemotoxicity but do not delay glioma progression. Exp. Gerontol. 2013; 48, 1120–1128.

Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, Dubeau L, Yap LP, Park R, Vinciguerra M, Di Biase S, Mirzaei H, Mirisola MG, Childress P, Ji L, Groshen S, Penna F, Odetti P, Perin L, Conti PS, Ikeno Y, Kennedy BK, Cohen P, Morgan TE, Dorff TB, Longo VD. A Periodic Diet that Mimics Fasting Promotes Multi-System Regeneration, Enhanced Cognitive Performance, and Healthspan.Cell Metab.2015; 7;22(1):86-99.

Brunet A. The multiple roles of FOXO transcription factors. Med Sci (Paris) 2004;20:856.

Bürkle A, Caselli G, Franceschi C, Mariani E, Sansoni P, Santoni A, Vecchio G, Witkowski JM, Caruso C: Pathophysiology of ageing, longevity and age related diseases. Immun Ageing. 2007;4:4

Cheng, C.-W., Adams, G.B., Perin, L., Wei, M., Zhou, X., Lam, B.S., Da Sacco, S., Mirisola, M., Quinn, D.I., Dorff, T.B., et al.. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. Cell Stem Cell 2014;14, 810–823.

Cahill GF Jr. Fuel metabolism in starvation. Annu Rev Nutr. 2006; 26:1–22.

Candore G, Balistreri CR, Colonna-Romano G, Grimaldi MP, Lio D, Listi' F, Scola L, Vasto S, Caruso C. Immunosenescence and anti-immunosenescence therapies: the case of probiotics. Rejuvenation Res. 2008;11(2):425-32**.**

Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Di Bona D, Duro G, Lio D, Matranga D, Pellicanò M, Rizzo C, Scapagnini G, Vasto S. Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in Alzheimer disease: therapeutic implications. Rejuvenation Res 2010;13:301-13.

Candore G, Caruso C, Colonna-Romano G. Inflammation, genetic background and longevity. Biogerontology 2010;11:565-73.

Cano PG, Santacruz A, Trejo FM, Sanz Y. Bifidobacterium CECT 7765 improves metabolic and immunological alterations associated with obesity in high-fat diet-fed mice. Obesity (Silver Spring). 2013; 21(11):2310-21.

Caruso C, Passarino G, Puca A, Scapagnini G. “Positive biology": the centenarian lesson. Immun Ageing 2012;9:5.

Capri M, Salvioli S, Monti D, Caruso C, Candore G, Vasto S, Olivieri F, Marchegiani F, Sansoni P, Baggio G, Mari D, Passarino G, De Benedictis G, Franceschi C. Human longevity within an evolutionary perspective: the peculiar paradigm of a post-reproductive genetics. Exp Gerontol 2008;43:53-60.

Capri M, Salvioli S, Sevini F, Valensin S, Celani L, Monti D, Pawelec G, De Benedictis G, Gonos ES, Franceschi C. The genetics of human longevity. Ann N Y Acad Sci 2006;1067: 252-63.

Carmody RN, Gerber GK, Luevano JM Jr, Gatti DM, Somes L, Svenson KL, Turnbaugh PJ. Diet dominates host genotype in shaping the murine gut microbiota.Cell Host Microbe. 2015; 14;17(1):72-84.

Chung H, Kasper DL. Microbiota-stimulated immune mechanisms to maintain gut homeostasis. Curr Opin Immunol. 2010;22(4):455-60.

Clarridge J. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 2004; 17: 840-862.

Claesson MJ, Cusack S, O'Sullivan O, et al. () Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 2011; 108, Suppl. 1, 4586–4591.

Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L, Schurmans S. The lipid phosphatase SHIP2 controls insulin sensitivity. Nature 2001;409:92–97.

Corona G, Spencer J, Dessi M: Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract. Toxicol Ind Health 2009;25:285-293.

Cummings, J.H. and Macfarlane, G.T. (1997) Role of intestinal
bacteria in nutrient metabolism. JPEN – J Parenter Enteral
Nutr 21, 357–365.

Deguchi, Y., Morishita,
Cummings, J.H. and Macfarlane, G.T. (1997) Role of intestinal bacteria in nutrient metabolism. JPEN – J Parenter Enteral Nutr 21, 357–365.
Deguchi, Y., Morishita,
Cummings, J.H. and Macfarlane, G.T. (1997) Role of intestinal
bacteria in nutrient metabolism. JPEN – J Parenter Enteral
Nutr 21, 357–365.

Cummings, J.H. and Macfarlane, G.T. Role of intestinal bacteria in nutrient metabolism. JPEN – J Parenter Enteral Nutr 1997; 21, 357–365.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014; 23;505(7484):559-63.

Deguchi, Y., Morishita, T. and Mutai, M. Comparitive studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agric Biol Chem 1985; 49, 13–19.

de la Torre-Carbot K, Chavez-Servin JL, Jauregui O, Castellote AI, Lamuela-Raventos RM, Fito M, Covas MI, Munoz-Aguayo D, Lopez-Sabater MC: Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: determination by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry. Anal Chim Acta 2007;583:402-410.

Delgado S, Sua´rez A & Mayo B. Identification of dominant bacteria in feces and colonic mucosa from healthy Spanish adults by culturing and by 16S rDNA sequence analysis 2006; Dig Dis Sci 51, 744–751.

Dethlefsen L, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. Trends Ecol. Evol. 2006. 21: 517-523.

Dorman JB, Albinder B, Shroyer T, Kenyon C. The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. Genetics 1995;141:1399-406.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science. 2005; 10;308(5728):1635-8.

Estruch R, Ros E, Salas-Salvadó J, Covas MI, Corella D, Arós F, Gómez-Gracia E, Ruiz-Gutiérrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pintó X, Basora J, Muñoz MA, Sorlí JV, Martínez JA, Favier C, Vaughan E, De Vos WM, et al. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 2002; 68, 219–226.

Ferreira IL, Resende R, Ferreiro E, Rego AC, Pereira CF. Multiple defects in energy metabolism in Alzheimer's disease. Curr Drug Targets 2010;11:1193–1206.

Flachsbart F, Caliebe A, Kleindorp R, Blanchè H, von Eller-Eberstein, Nikolaus S, Schreiber S, Nebel A. Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci U S A 2009;106:2700-5.

Fontana L, Klein S. Aging, adiposity, and calorie restriction. JAMA. 2007; 297:986–994
Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. Cell. 2015 Mar 26;161(1):106-18.

Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010;
328:321–326.

Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging Cell. 2008; 7:681–687.

Frisardi V, Solfrizzi V, Capurso C, Imbimbo BP, Vendemiale G, Seripa D, Pilotto A, Panza F. Is insulin resistant brain state a central feature of the metabolic-cognitive syndrome? J. Alzheimers Dis 2010;9:399–417.

Fujimoto T, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, Andoh A. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J Gastroenterol Hepatol. 2013; 28(4):613-9.

Gavini, F., Cayuela, C., Antoine, J.-M., Lecoq, C., Lefebvre, B., Membre, J.-M. and Neut, C. Differences in the spatial distribution of bifidobacterial and enterobacterial species in human faecal microflora of three different (children, adults, elderly) age groups. Microbial Ecol Health Dis 2001; 13, 40–45.

Gems D, McElwee JJ. Aging: Microarraying mortality. Nature 2003;424:259–261.

Gonidakis S, Finkel SE, Longo VD. Genome-wide screen identifies Escherichia coli TCA-cyclerelated mutants with extended chronological lifespan dependent on acetate metabolism and the hypoxia-inducible transcription factor ArcA. Aging Cell. 2010; 9:868–881.

Gonzalez-Santiago M, Fonolla J, Lopez-Huertas E: Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. Pharmacol Res 2010;61:364-370.

Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. Human genetics shape the gut microbiome. Cell. 2014;159(4):789-99.

Griffiths EA, Duffy LC, Schanbacher FL, et al. In vivo effects of bifidobacteria and lactoferrin on gut endotoxin concentration and mucosal immunity in Balb/c mice. *Digestive Diseases and Sciences*.2004;49(4):579–589.

Grolund MM, Lehtonen OP, Eerola E, et al. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr 1999;28, 19–25.

Guarner F & Malagelada JR . Gut flora in health and disease. Lancet 2003; 361, 512–519.

Harvie, M.N., Pegington, M., Mattson, M.P., Frystyk, J., Dillon, B., Evans, G., Cuzick, J., Jebb, S.A., Martin, B., Cutler, R.G., et al. (2011). The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women. Int J Obes (Lond) 35, 714–727.

Hine, C., Harputlugil, E., Zhang, Y., Ruckenstuhl, C., Lee, B.C., Brace, L., Longchamp, A., Trevin˜ o-Villarreal, J.H., Mejia, P., Ozaki, C.K., et al. Endogenous hydrogen sulfide production is essential for dietary restriction benefits. 2015. Cell 160, 132–14.

Honda Y, Honda S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mnsuperoxide dismutase gene expression in Caenorhabditis elegans. FASEB J 1999;13:1385–1393.

Honjoh S, Yamamoto T, Uno M, Nishida E. Signalling through RHEB-1 mediates intermittent fasting induced longevity in C. elegans. Nature. 2009; 457:726–730.

Hopkins, M.J., Sharp, R. and Macfarlane, G.T.. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut 2001; 48, 198–205.

Invidia L, Salvioli S, Altilia S, Pierini M, Panourgia MP, Monti D, De Rango F, Passarino G, Franceschi C. The frequency of Klotho KL-VS polymorphism in a large Italian population, from young subjects to centenarians, suggests the presence of specific time windows for its effect. Biogerontology 2010;11:67-73.

Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, Hill C, Gahan CG. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proceedings of the National Academy of Sciences of the United States of America. 2014;111:7421–7426.

Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, Kennedy BK, Kaeberlein M. Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging Cell. 2006; 5:487–494.

Khymenets O, Fito M, Covas MI, Farre M, Pujadas MA, Munoz D, Konstantinidou V, De la Torre R. Mononuclear cell transcriptome response after sustained virgin olive oil consumption in humans: an exploratory nutrigenomics study. OMICS 2009;13:7-19.

Kaisaki PJ, Delepine M,Woon PY, Sebag-Montefiore L, Wilder SP,Menzel S, Vionnet N,Marion E, Riveline JP, Charpentier G, Schurmans S, Levy JC, Lathrop M, Farrall M, Gauguier D. Polymorphisms in type II SH2 domain-containing inositol 5-phosphatase (INPPL1, SHIP2) are associated with physiological abnormalities of the metabolic syndrome. Diabetes 2004;53:1900–1904.
Kenyon C. The plasticity of aging: Insights from long-lived mutants. Cell 2005;120:449–460.

Koshiyama H. Explanation of the Insulin Paradox From the Evolutionary Point of View. Jpn Clin Med 2012;3:21–24.

Konstantinidou V, Covas M-I, Munoz-Aguayo D, Khymenets O, De la Torre R, Saez G, Tormos MdC, Toledo E, Marti A, Ruiz-Gutiierrez V, Ruiz Mendez MV, Fito M. In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB J 2010;24:2546-2557.

Kuro-o M. Klotho and aging. Biochim Biophys Acta 2009;1790:1049-58

Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Science. 2005 Sep 16;309(5742):1829-33.

Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH, Welling GW. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol.1995;61(8):3069-75

Lee, C., Raffaghello, L., and Longo, V.D.Starvation, detoxification, and multidrug resistance in cancer therapy. Drug Resist. Updat. 2012. 15, 114–122.

Lee GD, Wilson MA, Zhu M, Wolkow CA, de Cabo R, Ingram DK, Zou S. Dietary deprivation extends lifespan in Caenorhabditis elegans. Aging Cell. 2006; 5:515–524.

Li F, Jiang C, Krausz KW, Li Y, Albert I, Hao H, Fabre KM, Mitchell JB, Patterson AD, Gonzalez FJ. Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity. Nature communications. 2013;4:2384.

Longo VD, Ellerby LM, Bredesen DE, Valentine JS, Gralla EB. Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. J Cell Biol. 1997; 137:1581–1588.

Longo VD, Shadel GS, Kaeberlein M, Kennedy B. Replicative and chronological aging in Saccharomyces cerevisiae. Cell Metab. 2012; 16:18–31.

Longo, VD, and Mattson, M.P. Fasting: molecular mechanisms and clinical applications. Cell Metab 2014; 19, 181–192.

Longo VD, Finch CE. Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 2003; 299:1342–1346.

Lupp C, Robertson M, Wickham M, Sekirov I, Champion O, Gaynor E, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe.2007;2:119–29.

Macfarlane, G.T. and Gibson, G.R. Co-utilization of polymerized carbon sources by Bacteroides ovatus grown in a two-stage continuous culture system. Appl Environ Microbiol 1991; 57, 1–6.

Majumdar V, Nagaraja D, Christopher R. Association of the functional KL-VS variant of Klotho gene with early-onset ischemic stroke. Biochem Biophys Res Commun 2010;403:412-6.

Marques TM, Wall R, Ross RP, Fitzgerald GF, Ryan CA, Stanton C. Programming infant gut microbiota: influence of dietary and environmental factors. Curr Opin Biotechnol. 2010;21(2):149-56.

Mattson, M.P. Energy intake and exercise as determinants of brain health and vulnerability to injury and disease. Cell Metab 2012; 16, 706–722.

Martín R, Miquel S, Chain F, Natividad JM, Jury J, Lu J, Sokol H, Theodorou V, Bercik P, Verdu EF, Langella P,Bermúdez-Humarán LG. Faecalibacterium prausnitzii prevents physiological damages in a chronic low-grade inflammation murine model. BMC Microbiol. 2015; 21;15:67. doi: 10.1186/s12866-015-0400-1.

Masoro EJ. Overview of caloric restriction and ageing. Mech Ageing Dev. 2005; 126:913–922.

Million M, Maraninchi M, Henry M, Armougom F, Richet H, Carrieri P, Valero R, Raccah D, Vialettes B, Raoult D. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. International journal of obesity. 2012;36:817–825.

Million M, Angelakis E, Maraninchi M, Henry M, Giorgi R, Valero R, Vialettes B, Raoult D. Correlation between body mass index and gut concentrations of Lactobacillus reuteri, Bifidobacterium animalis, Methanobrevibacter smithii and Escherichia coli. International journal of obesity. 2013;37:1460–1466.

Moore WE, Moore LH. Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol. 1995 Sep;61(9):3202-7.

Novelli V, Viviani Anselmi C, Roncarati R, Guffanti G, Malovini A, Piluso G, Puca AA. Lack of replication of genetic associations with human longevity. Biogerontology 2008;9:85-92.

Omodei D, Fontana L. Calorie restriction and prevention of age-associated chronic disease. FEBS Lett 2011;585:1537–1542.

O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006; 7(7):688-93. Review.

O'Sullivan DJ. Methods for analysis of the intestinal microflora. Curr. Issues Intest. Microbiol. 2000; 1: 39-50.

O'Toole PW, Claesson MJ. Gut microbiota: Changes throughout the lifespan from infancy to elderly. Int Dairy J 2010; 20, 281-291.

Penders J, Thijs C, Vink C, et al. Factors influencing the composition of the intestinal microbiota in early
infancy. Pediatrics 2006; 118, 511–521.

Percival, R.S., Marsh, P.D. and Challacombe, S.J. Serum antibodies to commensal oral and gut bacteria vary with age. FEMS Immunol Med Microbiol. 1996; 15, 35–42

Piper MD, Partridge L. Dietary restriction in Drosophila: delayed aging or experimental artefact? PLoS Genet. 2007; 3:e57.

Pitozzi V, Jacomelli M, Catelan D, Servili M, Taticchi A, Biggeri A, Dolara P, Giovannelli L. Long-term dietary extra-virgin olive oil rich in polyphenols reverses age-related dysfunctions in motor coordination and contextual memory in mice: role of oxidative stress. Rejuvenation Res 2012;15:601-12.

Plum L, Schubert M, Bru¨ ning JC. The role of insulin receptor signaling in the brain. Trends Endocrino Metab 2005;16:59–65.

Porte D Jr, Baskin DG, Schwartz MW. Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. Diabetes 2005;54:1264–1276.

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010; 4;464(7285):59-65.

Raffaghello, L., Lee, C., Safdie, F.M., Wei, M., Madia, F., Bianchi, G., and Longo, V.D. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proc. Natl. Acad. Sci. USA 2008; 105, 8215–8220.

Blekhman R, Goodrich J, Huang K, Sun Q, Bukowski R, Bell J, Spector T, Keinan A, Ley R, Gevers D, Clark A. Host genetic variation impacts microbiome composition across human body sites Genome Biol. 2015; 16(1): 191.

Rolfe, R.D. Colonization resistance. In Gastrointestinal Microbiology ed. Mackie, R.I., White, B.A. and Isaacson, R.E 1997; 501–536.
Salminen A, Kauppinen A, Kaarniranta K. Emerging role of NF-κB signaling in the induction of senescence-associated secretory phenotype (SASP). Cell Signal 2012;24:835–845.

Salyers, A.A. Bacteroides of the human lower intestinal tract. Ann Rev Microbiol 1984; 38, 293–313.

Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010;11:76–83.

Scott KP, Duncan SH & Flint HJ. Dietary fibre and the gut microbiota. Nutr Bullet 2008; 33, 201–211.

Shi, Y., Felley-Bosco, E., Marti, T.M., Orlowski, K., Pruschy, M., and Stahel, R.A.. Starvation-induced activation of ATM/Chk2/p53 signaling sensitizes cancer cells to cisplatin. BMC Cancer. 2012; 12, 571.

Shaw, A.C., Joshi, S., Greenwood, H., Panda, A., and Lord, J.M. Aging of the innate immune system. Curr. Opin. Immunol. 2010; 22, 507–513.

Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, Benyamin FW, Lei YM, Jabri B, Alegre ML, Chang EB, Gajewski TF. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy Science. 2015 27;350(6264):1084-9.

Soeda Y, Tsuneki H, Muranaka H, Mori N, Hosoh S, Ichihara Y, Kagawa S, Wang X, Toyooka N, Takamura Y, Uwano T, Nishijo H, Wada T, Sasaoka T. The inositol phosphatase SHIP2 negatively regulates insulin/IGF-I actions implicated in neuroprotection and memory function in mouse brain. Mol Endocrinol 2010; 24:1965–1977.

Soerensen M, Dato S, Christensen K, McGue M, Stevnsner T, Bohr VA, Christiansen L. Replication of an association of variation in the FOXO3A gene with human longevity using both case-control and longitudinal data. Aging Cell 2010;9:1010-7.

Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. Adherence to Mediterranean diet and health status: meta-analysis. BMJ 2008;337-344.

Suh Y, Atzmon G, Cho MO, Hwang D, Liu B, Leahy DJ, Barzilai N, Cohen P. Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc Natl Acad Sci U S A 2008;105:3438-42.

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease–is this type 3 diabetes? J Alzheimers Dis 2005;7:63–80.

Stephen, A.M., Wiggins, H.S. and Cummings, J.H. Effect of changing transit time on colonic microbial metabolism in man. Gut 1987; 28, 601–609.

Tiihonen K, Ouwehand AC, Rautonen N. Human intestinal microbiota and healthy ageing. Ageing Res Rev. 2010;9(2):107-16.

Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012; 489:242–249.

Trichopoulou A, Orfanos P, Norat T, Bueno-de-Mesquita B, Ocké MC, Peeters PH, van der Schouw YT, Boeing H, Hoffmann K, Boffetta P, Nagel G, Masala G, Krogh V, Panico S, Tumino R, Vineis P, Bamia C, Naska A, Benetou V, Ferrari P, Slimani N, Pera G, Martinez-Garcia C, Navarro C, Rodriguez-Barranco M, Dorronsoro M, Spencer EA, Key TJ, Bingham S, Khaw KT, Kesse E, Clavel-Chapelon F, Boutron-Ruault MC, Berglund G, Wirfalt E, Hallmans G, Johansson I, Tjonneland A, Olsen A, Overvad K, Hundborg HH, Riboli E, Trichopoulos D. Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. BMJ 2005;330:991.

Van der Waaij, D., Berghuis de-vries, J.M. and Lekkerkerkvander Wees, J.E.C. Colonization resistance of the digestive tract in conventional and antibiotic treated mice. J Hyg 1971; 69, 405–411.

Vanughan EE, Schut F, Heilig HG, Zoetendal EG, de Vos WM, Akkermans AD.. A molecular view of the intestinal ecosystem. Curr. Issues Intest. Microbiol. 2000; 1: 1-12.

Verweij M, van de Ven M, Mitchell JR, van den Engel S, Hoeijmakers JH, Ijzermans JN, de Bruin RW. Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice. Transplantation. 2011; 15;92(7):752-8.

Walford RL. The 120-year diet. Am J Clin Nutr. 1988;47(1):162.

Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, Alatossava T
Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gelelectrophoresis and species-specific PCR primers. Appl Environ Microbiol. 2000;66(1):297-303.

Wang X, Heazlewood SP, Krause DO, Florin TH.
Molecular characterization ofthe microbial species that colonize human ileal and col onic mucosa by using 16S rDNA sequence analysis. J Appl Microbiol. 2003;95(3):508-20.

Wasselin T, Zahn S, Maho YL, Dorsselaer AV, Raclot T, Bertile F. Exacerbated oxidative stress in the fasting liver according to fuel partitioning. Proteomics. 2014 Aug;14(16):1905-21.

Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L, Longo VD. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. 2008; 4:e13.

Weindruch R, Naylor PH, Goldstein AL, Walford RL Influences of aging and dietary restriction on serum thymosin alpha 1 levels in mice. J Gerontol 1988;43:B40–B42.

Woodmansey E. Intestinal bacteria and ageing. J Appl Microbiol 2007 102, 1178–1186.

Woodmansey, E.J., McMurdo, M.E., Macfarlane, G.T. and Macfarlane, S. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in non-antibiotic-treated elderly subjects. Appl Env Microbiol 2004; 70, 6113–6122.

Wozniak M, Rydzewski B, Baker SP, Raizadai M. The cellular and physiological actions of insulin in the central nervous system. Neurochem Int 1993;22:1–10.

Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD. FOXO3A genotype is strongly associated with human longevity. Proc Natl Acad Sci U S A 2008;105:13987-92.

Xie L, Gong YY, Lian SG, Yang J, Yang Y, Gao SJ, Xu LY, Zhang YP. Absence of association between SNPs in the promoter region of the insulin-like growth factor 1 (IGF-1) gene and longevity in the Han Chinese population. Exp Gerontol 2008;43:962-5.

Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. Nature 2012; 486, 222–227.

Zarrinpar, A., Chaix, A., Yooseph, S., and Panda, S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. Cell Metab. 2014; 20, 1006–1071.

Ziv E, Hu D. Genetic variation in insulin/IGF-1 signaling pathways and longevity. Ageing Res Rev 2011;10:201-4.

Ringraziamenti

Vorrei ringraziare la Prof.ssa Candore e il Prof. Caruso per avermi guidata, aiutata e spronata durante tutti questi tre anni.

Un ulteriore ringraziamento va a Valter Longo che mi ha dato l'opportunità di svolgere una indimenticabile esperienza professionale e di vita all' "University of Sauthern California di Los Angeles".

Infine ringrazio i miei genitori per avermi trasmesso la virtù dell'impegno del lavoro e del coraggio nell' inseguire i propri sogni.