UNIVERSITÀ DEGLI STUDI DI PALERMO

dibimef
Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi

Sezione di Patologia Generale

Insulin pathway and its correlation with ageing and longevity

Dottorato di ricerca in Biopatologia
XXIV Ciclo
SSD MED/04

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

1. **Accardi G**, Caruso C, Colonna-Romano G, Camarda C, Monastero R, Candore G. Can Alzheimer disease be a form of type 3 diabetes? Rejuvenation Res. 2012;15:217-21.

   Abstract

   Alzheimer disease (AD) and metabolic syndrome are two highly prevalent pathological conditions of Western society due to incorrect diet, lifestyle, and vascular risk factors. Recent data have suggested metabolic syndrome as an independent risk factor for AD and pre-AD syndrome. Furthermore, biological plausibility for this relationship has been framed within the "metabolic cognitive syndrome" concept. Due to the increasing ageing of populations, prevalence of AD in Western industrialized countries will rise in the near future. Thus, new knowledge in the area of molecular biology and epigenetics will probably help to make an early molecular diagnosis of dementia. An association between metabolic syndrome and specific single-nucleotide polymorphisms (SNPs) in the gene INPPL1, encoding for SHIP2, a SH2 domain-containing inositol 5-phosphatase involved in insulin signaling, has been described. According to recent data suggesting that Type 2 diabetes represents an independent risk factor for AD and pre-AD, preliminary results of a case-control study performed to test the putative association between three SNPs in the SHIP2 gene and AD show a trend toward association of these SNPs with AD.

2. Di Carlo M, Giacomazza D, Picone P, Nuzzo D, Vasto S, **Accardi G**, Caruso C, San Biagio P.L. A close connection: Alzheimer's disease and type 2 diabetes. Curr. Topics Biochem. Res. 2012; 14:1-13.

   Abstract

   In the recent years a growing body of evidence links insulin resistance and insulin action to neurodegenerative diseases, especially Alzheimer's disease (AD). The importance of insulin in ageing as well as its role in cognition and other aspects of normal brain functions are well established. The hippocampus and cerebral cortex-distributed insulin and insulin receptor (IR) have been shown to be involved in brain cognitive functions. Conversely, deterioration of IR signaling is involved in ageing related brain degeneration such as in AD and cognitive impairment in type 2 diabetes patients. Insulin administration, while maintaining euglycemia, improves memory in both healthy adults and Alzheimer's disease patients. In the present review, some common links between AD and type 2 diabetes are presented. Furthermore, several biochemical aspects existing in both pathologies are highlighted.

3. Balistreri CR, Candore G, **Accardi G**, Bova M, Buffa S, Bulati M, Forte GI, Listì F, Martorana A, Palmeri M, Pellicanò M, Vaccarino L, Scola L, Lio D, Colonna-Romano G. Genetics of longevity. data from the studies on Sicilian centenarians. Immun Ageing. 2012; 9:8.

   Abstract

   The demographic and social changes of the past decades have determined improvements in public health and longevity. So, the number of centenarians is increasing as a worldwide phenomenon.

Scientists have focused their attention on centenarians as optimal model to address the biological mechanisms of "successful and unsuccessful ageing". They are equipped to reach the extreme limits of human life span and, most importantly, to show relatively good health, being able to perform their routine daily life and to escape fatal age-related diseases, such as cardiovascular diseases and cancer. Thus, particular attention has been centered on their genetic background and immune system. In this review, we report our data gathered for over 10 years in Sicilian centenarians. Based on results obtained, we suggest longevity as the result of an optimal performance of immune system and an over-expression of anti-inflammatory sequence variants of immune/inflammatory genes. However, as well known, genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Epigenetics is associated with ageing, as demonstrated in many studies. In particular, ageing is associated with a global loss of methylation state. Thus, the aim of future studies will be to analyze the weight of epigenetic changes in ageing and longevity.

4. 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 [1]. 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 [2,3]. 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 [4].

5. Incalcaterra E, **Accardi G**, Balistreri CR, Caimi G, Candore G, Caruso M, Caruso C. Pro-inflammatory genetic markers of atherosclerosis. Curr Atheroscler Rep. 2013;15:329.

Abstract

Atherosclerosis (AS) is a chronic, progressive, multifactorial disease mostly affecting large and medium-sized elastic and muscular arteries. It has formerly been considered a bland lipid storage disease. Currently, multiple independent pathways of evidence suggest this pathological condition is a peculiar form of inflammation, triggered by cholesterol-rich lipoproteins and influenced both by environmental and genetic factors. The Human Genome Project opened up the opportunity to dissect complex human traits and to understand basic pathways of multifactorial diseases such as AS. Population-based association studies have emerged as powerful tools for examining genes with a role in common multifactorial diseases that have a strong environmental component. These association studies often estimate the risk of developing a certain disease in carriers and non-carriers of a particular

genetic polymorphism. Dissecting out the influence of pro-inflammatory genes within the complex pathophysiology of AS and its complications will help to provide a more complete risk assessment and complement known classical cardiovascular risk factors. The detection of a risk profile will potentially allow both the early identification of individuals susceptible to disease and the possible discovery of potential targets for drug or lifestyle modification; i.e. it will open the door to personalized medicine.

6. Balistreri CR, Candore G, **Accardi G**, Colonna-Romano G, Lio D. NF-κB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies. Immun Ageing. 2013;10:24.

Abstract

Chronic inflammation is a major biological mechanism underpinning biological ageing process and age-related diseases. Inflammation is also the key response of host defense against pathogens and tissue injury. Current opinion sustains that during evolution the host defense and ageing process have become linked together. Thus, the large array of defense factors and mechanisms linked to the NF-κB system seem to be involved in ageing process. This concept leads us in proposing inductors of NF-κB signaling pathway as potential ageing biomarkers. On the other hand, ageing biomarkers, represented by biological indicators and selected through apposite criteria, should help to characterize biological age and, since age is a major risk factor in many degenerative diseases, could be subsequently used to identify individuals at high risk of developing age-associated diseases or disabilities. In this report, some inflammatory biomarkers will be

discussed for a better understanding of the concept of biological ageing, providing ideas on eventual working hypothesis about potential targets for the development of new therapeutic strategies and improving, as consequence, the quality of life of elderly population.

7. 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. 2013. [Epub ahead of print].

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.

8. 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. 2013. [Epub ahead of print].

   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.

9. **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. 2013. [Epub ahead of print].

   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.

10. Balistreri CR, **Accardi G**, Buffa S, Bulati M, Martorana A, Candore G, Colonna-Romano G, Lio D, Caruso C. Centenarian Offspring: a model for Understanding Longevity. Curr Vasc Pharmacol. 2013. [Epub ahead of print].

    Abstract

    A main objective of current medical research is the improving of life quality of elderly people as priority of the continuous increase of ageing population. This phenomenon implies several medical, economic and social problems because of dramatic increase in number of not autonomous individuals affected by various pathologies. Accordingly, the research interest is focused on understanding the biological mechanisms involved in determining the positive ageing phenotype, i.e. the centenarian phenotype. In achieving this goal the choice of an appropriate study models is fundamental. Centenarians have been used as an optimal model for successful ageing. However, it is characterized

by several limitations, i.e. the selection of appropriate controls for centenarians and the use itself of the centenarians as a suitable model for healthy ageing. Thus, the interest has been centered on centenarian offspring, healthy elderly people. They may represent a model for understanding exceptional longevity for the following reasons: to exhibit a protective genetic background, cardiovascular and immunological profile as well as a reduced rate of cognitive decline than age-matched people without centenarian relatives. Several of these aspects are summarized in this review based on the literature and the results of our studies.

11. 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. 2013. [Epub ahead of print].

    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.

12. Balistreri CR, **Accardi G**, Candore G. Probiotics and Prebiotics: Health Promotion by Immune Modulation in the Elderly. In “Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases”. Edited by Ronald Ross Watson and Victor R. Preedy. 2013; pp 257-269.

    Excerpta

Human subjects may be described as ‘metaorganisms’ because of their close symbiotic relationship with indigenous gut microbiota (Turnbaugh et al., 2007). This postulation proposes aging as the result of a cross-talk between environment, intestinal microbiota, and host immune system. Among these, the gut microbiota plays the principal role, in addition to maintaining human health (Chung and Kasper, 2010). 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).

13. **Accardi G,** Balistreri CR, Caruso C, Candore G. Diet and Immunosenescence. In “Immunology of Aging”. Edited by Springer. 2014; pp 285-293.

    Ageing is a systemic condition leading to a gradual loss of molecular and cellular fidelity. A feature of ageing is immunosenescence, consisting in several modifications that increase morbidity and mortality in elderly. Environment, genetic background, immune system, and intestinal microbiota play a fundamental role in immunosenescence. The development of a chronic, low-grade,

inflammatory status, known as “inflamm-ageing,” is a typical aspect of immunosenescence mostly due to the pro-inflammatory cytokine production linked to the chronic antigenic load. Nutrition can act on ageing, immunity, and health in general. Unbalanced diet with an insufficient intake of micro- and macronutrient and vitamins is a major nutritional problem among elderly, resulting in a dramatic change in gut microbiota. Calorie restriction and long-term adherence to Mediterranean diet could prevent or manage age-related diseases and immunosenescence.

14. Balistreri CR, **Accardi G,** Caruso C, Candore G. Biomarkers and Inflammatory Network in Aging: Targets for Therapies. In “Inflammation, advancing age and nutrition”. Edited by Elsevier. 2014; pp. 1-11.

    Excerpta

    Aging is recognized as a complex process, induced by intricate interactions between genetic, epigenetic, stochastic, and environmental factors. These factors contribute to a loss of molecular fidelity that results from the random accumulation of damage (particularly to nuclear and mitochondrial DNA) at the cellular, tissue, and organ levels and/or to the whole body, compatible with the “disposable soma” theory of aging [1]. This theory states that both the architecture and functioning of physiological processes and regulatory (*immune and endocrine*) systems are modified during aging, which leads to a deterioration of homeostatic capacity.

LIST OF ABBREVIATION

AD Alzheimer's diseases

APO Apolipoprotein

Bcl-3 B-cell CLL/lymphoma 3

CAMKIV Calcium/calmodulin-dependent protein kinase IV

CO Centenarian offspring

CVD Cardiovascular disease

EVOO Extra virgin olive oil

FOXO Forkhead box O

GSK3 Glycogen synthase kinase 3

GWAS Genome wide association study

HDL High density lipoprotein

HSP Heat shock protein

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

LDL Low density lipoprotein

LLI Long lived individuals

MI Myocardial infarction

MUFA Monounsatured fatty acid

MD Mediterranean diet

MS Metabolic syndrome

NF-kB Nuclear factor kappa beta

OR Odd ratio

PD Parkinson's disease

PI3K Phosphoatidyl inositol 3-kinase

PIP2 Phosphoatidyl inositol 2-phosphate

PIP3 Phosphoatidyl inositol 3-phosphate

RAS Rat sarcoma protein

SASP Senescence-associated secretory phenotype

SHIP2 Src homology domain-containing inositol 5-phosphatase 2

SIRT1 Silent mating type information regulation 1

SNP Single nucleotide polymorphism

SOD Superoxide dismutase

T2DM Type 2 diabetes mellitus

TF Transcription factor

TLR Toll like receptor

1. INTRODUCTION

1.1 AGEING AND LONGEVITY

Ageing is a complex phenomenon or "trait" than cannot be exhaustively defined. Thus an integrated approach (biologic, demographic, antropologic and historic) is needed to try to understand the different ageing processes. Ageing is unavoidable and leads to the reduction of the ability to adapt to the environment, involving the organism at all levels (DNA, cells, tissues and whole systems). The "onset" of ageing is due to the loss of molecular fidelity that varies from subject to subject, in terms of rate and time of onset. Some phenotypic features of the old are not dangerous or life threatening whereas others are life threatening and increase the vulnerability leading to death (**Table 1**) (*Troen 2003*).

Table 1. FEATURES OF AGEING. The loss of molecular precision causes wide range of age-related modifications.

– Changes in cells, tissues and organs after maturation
– Progressive reduction in physiologic function of tissues and organs
– Reduced ability to respond to environmental stimuli due to the loss of homeostasis
– Increased susceptibility and vulnerability to pathologies
– Increased risk of death

Not dangerous: grey hair, liver spots, presbyopia, hearing loss, short memory loss and increase of time of reaction.
Potentially dangerous: molecular changes of cells, tissues and organs that increases the susceptibility to cancer, ictus, cardiovascular diseases, Alzheimer's disease and Parkinson's disease.

In 2012, a group of scientists, drafted a panel statement to summarize, clarify and highlight features of ageing, longevity and exceptional longevity, three phenotypes that are spreading rapidly worldwide. They analyzed the ageing theories taking into account the evolutionary perspective and listed the biomarkers associated with the three phenotypes. On these basis, they discussed on the possible treatments to counteract or slow down ageing (**Box 1**) (*Avery et al 2013*).

The difference among people has a multifactorial origin depending on genetic background, stochastic and environmental variables with a strong component dependent on the life-style (*Mitnitski et al 2001; Kirkwood 2005*). Ageing results in compromised stress response, greater homeostatic imbalance and elevated risk of disease (*Rakyan et al 2010*). All these elements lead people to reach different ages in different conditions, therefore different life-span. Ageing itself is associated with progressive homeostatic/homeodynamic dysregulation that makes the organism less, and eventually, non-resilient (*Yates 2002; Lipsitz 2004*). This leads to the incapacity to adapt to stress and to a decline in functional capacity.

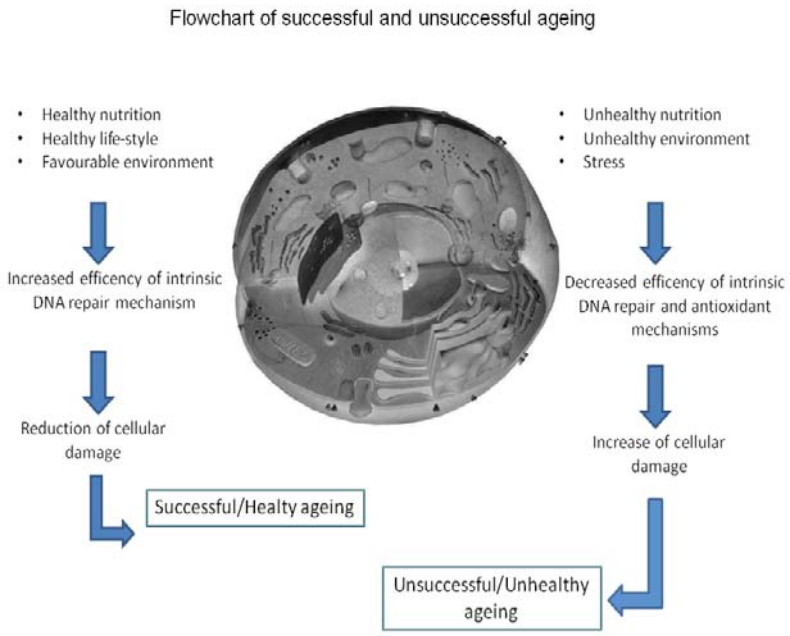
The ageing condition itself changes the performance of physiological systems and increases the susceptibility to death but new evidences suggest that the process is modifiable thus becoming possible to delay age-related diseases en bloc (*Fontana et al 2010*) (See below).

There are two main ways to become old: with success (successful ageing) and without success (unsuccessful or pathological ageing) (**Figure 1**). This latter is manifested by people that from 60 years old, develop one or more age-related diseases: neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), metabolic diseases such as

metabolic syndrome (MS) and type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVDs), cancer.

On the other hand, the successful ageing is represented by centenarian people since most subjects reach the age of 100 or more without any age-related disease, in good physical and mental condition. They represent the best model to study successful ageing and longevity although they have different genetic features and life-style thus, borrowing a concept from physics, they cannot be considered a “close system”.

Figure 1. FLOWCHART OF SUCCESSFUL AND UNSUCCESSFUL AGEING. Random molecular damage determines the accumulation of cellular defects, hence causing frailty, disability and onset of age-related pathologies. Nutrition, life-style and environmental stimuli act on the efficiency of DNA repair mechanisms leading to successful or unsuccessful ageing.



Therefore, demographic selection has permitted to identify the centenarians as healthy survivors thus offering a “natural” selected population in which studying the effect of specific polymorphisms and genetic loci associated or not associated with longevity.

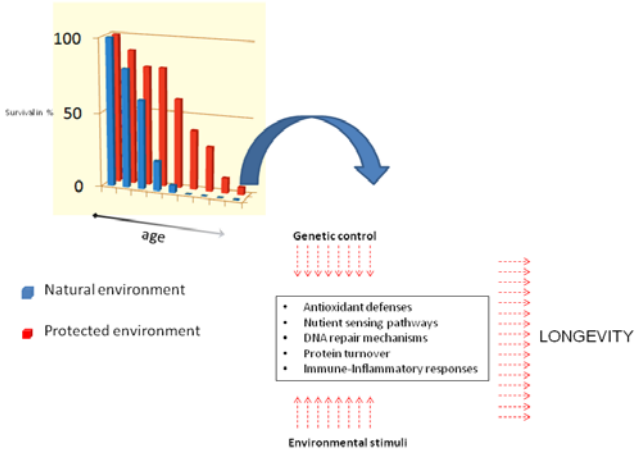
Undoubtedly, all these individuals are able to respond very well to the stressors and to repair damages thanks to “positive genes” involved in many cell functions (**Table 2**).

Table 2. EVOLUTION, AGEING AND LONGEVITY.

– The damage is intrinsic in life
– The loss of maintenance and repair systems are the evolutive causes of ageing
– Longevity can be regulated by genes involved in repair and maintenance processes such as genes involved in growth, oxidative stress, apoptosis, cell cycle, DNA repair, metabolism and immune-inflammatory response

Many of these, highlighted by association studies of candidate genes, revealed a wide range of data but not always repeatable (*Capri et al 2006*). This is probably due to the different genetic combination, the “genetic mosaic”, and the interaction with the environment (**Figure 2**).

Figure 2. RATE OF SURVIVAL IN DIFFERENT ENVIRONMENTS. The histogram shows the survival rate (from 0 to 100%) in natural and in protected environment with age. The reaching of longevity is under both genetic and environmental control and depends on the correct action of antioxidant defenses, DNA repair mechanisms and protein turnover.



The increased ability to reach the age of 100 in Westernized countries over the last 150 years and the reduction in the overall mortality clearly reflect the improvement of hygienic condition, thus the reduced exposure to infection and inflammation; the treatment of infectious diseases that has reduced both child and maternal deaths; the improvement in general of the quality of life; the attention to the diet and the advent of preventive medicine (*Kirkwood 2005*). During the last 2 centuries their number has grown and the average life span has increased at a rate of approximately 3 months/year in both sexes (*Oeppen et al 2002*). Obviously, the increase of average life-span has raised the number of nonagenarians (people that reach 90 or more years) and centenarians. In the USA, it has gone from 3,700 in 1940 to approximately 61,000 centenarians in 2006 (*Sonnega 2006*) In Italy and in a small area of Sicily, in the Sicani Mountain, the centenarian figures are higher, 2.4/10,000 and 10.37/10,000 respectively (*Vasto et al 2012a*).

These differences are probably due to life-style, healthier in Italy and in Sicily that in USA. Looking at the Italian ratio of centenarians per inhabitants, in some zones of Sicily there is more than a four-fold increase.

Since Sicilian population genetic structure is very homogeneous, in particular in the countryside, the explanation for these data probably resides in the environmental characteristics of the study sample. In particular, the area of Sicani Mountain was extensively studied in its dietary habits leading to the conclusion that this high rate of centenarians is strictly related to the adherence to the Mediterranean diet (MD) (*Vasto et al 2012a; Vasto et al 2012b)*. As we discussed in our papers (*Virruso et al 2013; Accardi et al 2014*), the MD consists in plant foods (fruit, vegetables, legumes, wholemeal bread and other forms of cereals, nuts and seeds), fresh fruit, olive oil as the principal source of fat, dairy products (principally cheese and yogurt), and poultry consumed in low to moderate amounts (fish only by coast inhabitants), zero to four eggs weekly, red meat in very low amounts, and wine in low to moderate amounts, normally during meals. This diet is hypocaloric with no sweeteners and sweet beverages and a low intake of animal proteins (*see the papers for references, abstract n°8 and n°13*).

However, a "favourable" genetic background is essential to live longer (*Balistreri et al 2012; Balistreri et al 2013, abstract n°10; Incalcaterra et al 2013*). Indeed, siblings and offspring of centenarian (CO), but not their spouses, show an increased odd ratio (OR) between 4- and 17-fold for longevity compared with appropriate controls thus they have a good chance to live approximately 100 years or over compared with the average population. CO have an advantageous genetic background characterized by favourable alleles, i.e. in Apolipoprotein (APO) C3 promoter (homozygosity for the -641 C or rs2542052), by a reduction in telomere attrition associated

with the presence of synonymous and intronic variants in human-telomerase reverse transcriptase gene, and heteroplasmic T152C variant in mt-DNA.

Protective genetic background, immunological profile and clinical history seem to be exhibited by CO when compared with age-matched people without centenarian relatives. They show a favorable lipid, immunological and cardiovascular profile and a decreased cognitive decline (lower serum levels of APOC3, Heat shock protein (Hsp) 70 and low density lipoprotein (LDL) C, and higher amount of high density lipoprotein (HDL) C, all features that consent them to escape morbidity and mortality for pathological events, including atherothrombosis, myocardial infarction, stroke and heart failure (*see the papers for references, abstract n°3, n°5 and n°10*).

However, from the evolutionary point of view, longevity is not a random process but is a stage that depends of the residual physiological functions after reproduction. Ageing is dependent by stochastic events and the ageing phenotype occurs after the accumulation of cellular damages that cannot be repaired at all by exhausted cellular systems. Thus longevity depends on the survival after reproduction and genes that lead to longevity are, for this theory, “survival genes” rather than “longevity genes” (*Hayflick 2007*).

Box 1. PANEL STATEMENT SUMMARY. Summary of the statement for ageing, longevity, exceptional longevity and related genetic and non genetic markers.

Ageing:
causes- breakdown of self organizing system and reduced ability to adapt to the environment;
where- primarily in economically developed countries;
what- process that increases the vulnerability and leads to death;
news- chronological and biological ageing: two sides of the same coin. They do not necessary have correspondence intra and inter individuals because of the different rate of ageing of tissues and organs of the same body;
contributing factors- cultural, anthropological, socio-economic status, sex, gender, ethnic differences, healthcare, environmental status, genetics, life occupation, stochastic events;
successful ageing: avoidance (or late onset) of age-related diseases and disability, preservation of desirable cognitive and physical function and social activities.

Longevity:
definitions- no consensus definition has been established;
exceptional longevity- defined in *relative* and *absolute* terms:
"relative": longevity is concept country/population specific and must take into consideration the life expectancy of the different populations/countries, which show great variability owing to historical, anthropological and socio-economic differences. *"Absolute"*: longevity could be defined according to the maximum lifespan attained and scientifically validated by human beings in the planet;
familial longevity: family with at least two living members aged $\geq$ 90 (long lived individuals (LLI), based on demographic data in Europe);

LLI study limits: enrolment of large number of phenotypically well characterized long living people (centenarians) thus small cohort groups; validation in different cohorts; enrolment of matched controls (same time but different life span duration); lack of information (environmental factors, lifestyle, quality of life, presence and duration of disabilities and diseases).;

Genetics in ageing and longevity:
Genes involved: genes that take part in: nutrient-sensing pathways, e.g. insulin/insulin-like growth factor (IGF-1), nutrient-sensing (mTOR); oxidative stress and anti-oxidant systems; control of immune-inflammatory responses; lipid metabolism and mitochondrial DNA (mtDNA).
Epigenetic changes caused by environmental factors (diet, life style, emotional stress, physical activity).

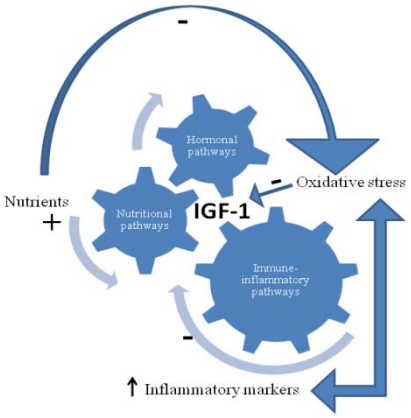
Study strategies in ageing/longevity
Gene knock down; human genetic population-based studies, family-based studies, "omics" studies, calorie restriction, hormonal replacement, antioxidant treatments, engineered negligible senescence, nucleic acid therapy, gene cloning, prevention of vascular events, cancer screening, healthy life style.

Goal: decrease morbidity and mortality associated with ageing, increase healthy life span.

1.2 INSULIN/IGF-1 AND NF-kB PATHWAYS: KEY ROLE IN AGEING AND LONGEVITY

The correlation between nutritional, hormonal and immune-inflammatory pathways is particularly evident in ageing and longevity (**Figure 3**).

Figure 3. CORRELATION BETWEEN NUTRITIONAL, HORMONAL AND IMMUNE-INFLAMMATORY PATHWAYS IN AGEING AND LONGEVITY. Insulin-like growth factor (IGF) 1constitutes the perfect link between nutritional, hormonal and immune-inflammatory pathways. IGF-1 levels are related to inflammatory markers, oxidative stress and specific nutrients.



In particular, we focused our attention on insulin/insulin-like growth factor (IGF) 1 and the downstream nuclear factor kappa beta (NF-kB) signaling cascades.

The insulin/IGF-1 signaling cascade starts from the binding to insulin or IGF-1 to the insulin or IGF-1 receptor. Consequently, inside the cell, the intracellular substrate proteins, known as insulin responsive substrates (IRSs), act as mediators for the intracellular effect of insulin, binding specific src-homology-2 domain proteins, which include important

enzymes such as phosphatidylinositol 3-kinase (PI3K) and other intracellular signaling systems, e.g. the adaptor protein growth factor receptor-bound protein 2 which connects with the rat sarcoma protein (RAS) pathway. PI3K activates the via of the second messenger phosphoatidyl inositol 3-phosphate (PIP3) leading to the activation of AKT, that acts on glycogen synthase kinase 3 (GSK3). In the brain, the deregulation of GSK3 activity determines neuronal cell death, hyperphosphorylation of tau protein and the production of amyloid protein.

Moreover AKT promotes the translocation of glucose transporter proteins (GLUT). The pathway activates transcription factors (TFs) and stimulates the growth promoting actions of insulin. Thus broadly, PI 3-kinase mediates insulin metabolic effects, e.g. cellular glucose uptake, while RAS significantly mediates insulin mitogenic effects (*see the papers for references, abstract n°2*).

On the other hand, AKT can also stimulates the NF-kB signaling activating the inhibitor of kB kinase (IkK) complex (IkK alpha e beta). The NF-kB pathway is involved in immune inflammatory mechanisms that can result in both positive or negative effects (*Gilmore et al 2012; Newton et al 2012*). NF-kB components (p50/p105 and p52/p100) and the members of the Rel family (RelA/p65, c-Rel and RelB) form dimers in the cytoplasm that are linked to the inhibitor of kappa B (IkB) proteins (IkBalpha, IkBbeta, IkBgamma, Ikbepsilon and B-cell CLL/lymphoma 3, known as Bcl3). The complex activation can be evocated both by immune insults and external and internal danger signals associated with cell senescence and ageing process, such as oxidative and genotoxic stress, tissue injuries and DNA damages. When specific immune receptors, such as Toll-like receptors (TLRs) and cytokine receptors, transduce the upstream signals, the kinases,

mainly IkKbeta, phosphorilate the IkB proteins which are released from the complex and then degraded by the proteasome. This leads to the translocation of NF-kB to the nucleus. Its binding to the DNA triggers the transcription of a number of genes including pro-inflammatory cytokines, chemokines, adhesion molecules, eicosanoids, growth factors, metallo-proteinases, nitric oxide, etc (*Gilmore et al 2012*).

Emerging experimental data principally performed on human skin fibroblasts have convincingly demonstrated the role of NF-κB signaling as the major pathway stimulating the senescence-associated secretory phenotype (SASP) (*Chien et al 2011; Rovillain et al 2011; Salminen et al 2012*).

The cellular senescence consists in a state of irreversible cell cycle arrest even if a mitotic stimulus occurs caused by stresses that are potentially oncogenic. Senescent cells are apoptosis resistant and acquire different functions and features respect to the cell line group. DNA damages, oncogenes expression, oxidative stress and mitogenic signals are some of the stresses that cause this condition (*Ben-Porath et al 2005; Campisi et al 2005; Lombard et al 2005; Braig et al 2006; Collado et al 2006; Campisi et al 2007*). Interestingly, senescent cells remain metabolic active releasing pro-inflammatory factors that determines the occurrence of the SASP. Emerging data has revealed that NF-κB signaling is the major signaling pathway which stimulates the appearance of SASP. With advancing age, increased cellular and tissues injuries determine a sustained NF-κB activation. In turn, SASP, found in several cells, such as fibroblasts, epithelial cells, endothelial cells, astrocytes, preadipocytes, leukocytes and postmitotic cells contributes, together with inflammageing, to the low chronic inflammation condition and consequently to the establishment of the

negative feedback typical of ageing process and to the onset of inflammatory age-related diseases (*Salminen et al 2010; Salminen et al 2012*). Indeed, the SASP of astrocytes, has been suggested to initiate or contribute to neuroinflammation, responsible of many neurodegenerative diseases, such as AD (*Campisi et al 2011*).

Many genetic mutations, in particular single nucleotide polymorphisms (SNPs), that extend life-span act on nutrient-sensing pathways such as insulin/IGF. A common evolutionary origin of ageing (meaning "survival" from the evolution) regulation probably exists. Indeed, models organisms, from yeast to mammals, highlight the role of these pathways in the modulation of life-span (*Longo et al 2003*). This may be due to the effect of the attenuation of these cascades that mimic calorie restriction, process well known to be involved in life-span extension from almost 80 years (*McCay et al 1935; Weindruch et al 1988*). In these models, calorie restriction, causing life-span extension and IGF-1 signaling reduction, is associated with decreased IGF-1 circulating levels (*Fontana et al 2008; Fontana et al 2010*).

IGF-1, IGF-1 receptor (IGF-1R), Forkhead box O (FOXO) 3A, Silent mating type information regulation 1 (SIRT1) and KLOTHO, all molecules directly or indirectly involved in insulin/IGF-1 pathway, are under reflectors of the ageing and longevity research 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 life-span 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 to date are not

conclusive (*Suh et al 2008; Xie et al 2008; Bonafè et al 2003; Albani et al 2009; Barbieri et al 2012*) .

The IGF-1 pathway downstream TF, FOXO3A, has also been extensively studied for its role in longevity, as mentioned above. This gene belongs to the FOXO family and encodes a TF with the typical domain of this family, the forkhead box, a conserved DNA-binding domain. It is one of the orthologue 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, identified as anti-ageing molecules in model organisms. SIRT1, one of the seven human sirtuin isoforms, called SIRT1-SIRT7, deacetylates FOXO3A modulating its response to oxidative stress (*Brunet 2004*). 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 gene KLOTHO, aptly named after one of the Greek goddesses Fates, 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 fifteen years ago, as a gene which, if knocked out in mice, precipitates their accelerated ageing, including short lifespan, while its over-expression suppresses ageing and extends lifespan (*Kuro-o 2009; Wang et al 2009*). On this basis, some human studies sought to demonstrate an association between the functional variant of KLOTHO “KL-VS” and ageing and longevity. This variant is a stretch that contains six polymorphisms 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*).

The Src homology domain-containing inositol 5-phosphatase 2 (SHIP2), has an important role in insulin pathway thus presumably in ageing and longevity but surely in age-related diseases. To regulate cellular levels of lipid secondary messengers such as PIP3, cells use two major classes of phosphoinositide phosphatases—the inositol polyphosphate 3-phosphatase PTEN and the SH2 domain-containing inositol 5-phosphatases 1 and 2 (SHIP1 and SHIP2) (*Dyson et al 2005*).

SHIP2 is a protein encoded by the gene inositol polyphosphate phosphatase-like 1 (INPPL1) that 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 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 features (*Kaisaki et al 2004*), finding partly confirmed by another study (*Kagawa et al 2005*). Moreover, a study conducted in non-T2DM subjects with hypertension found no association, identifying the T2DM as condition probably necessary for the association (*Marcano et al 2007*).

1.3 AGE RELATED DISEASES

As mentioned above, MS, T2DM and AD are classified as age-related diseases.

Human ageing and age-associated diseases are becoming one of the biggest challenges faced by developed and developing countries. In fact, the overall increase in average life expectancy is far greater than that for healthy life expectancy, as evidenced by the incremental burden of age-associated diseases, including cardiovascular disease, diabetes, hypertension and cancer. The financial burden caused by these chronic diseases is already overwhelming the healthcare and welfare systems of developed nations, and if present trends continue, the challenges could cause even larger problems.
MS represents a cluster of metabolic factors, such as IR due to an impairment in insulin signaling pathway, abdominal obesity, glucose intolerance, hypertension, hyperinsulinemia, and raised fasting plasma glucose, all conditions related to an increased risk for T2DM, AD and CVDs (*Wilcox 2005*; *Craft 2009; Sanz et al 2009; Ahtiluoto et al 2010; Elks et al 2010; Luchsinger 2010; Di Carlo et al 2012*). T2DM, the non-insulin dependent diabetes, is a lifelong, chronic, disease and is the most common form of diabetes account for 90–95% of all cases of diabetes (*Zimmet 1999; Zimmet et al 2001*).

It is characterized mainly by IR with hyperglycemia. To date, many evidences exist about the association between T2DM and AD that has as link factor the IR. IR can be manifested in peripheral tissues or directly in the brain as an “insulin resistance brain state” that contributes to cognitive impairment and neurodegeneration (*Frisardi et al 2010*). When glucose accumulates in the blood, determine hyperglycemia and hyperinsulinemia.

Hyperglycemia induces an increase of the peripheral use of insulin, which results in a reduction of insulin disposable for the brain and consequently in the alteration of tau and amyloid protein processing (*Plum et al 2005; Wozniak et al 1993*).

AD is the most common form of dementia, accounting for more than 50% of all cases of dementia. It is a neurodegenerative disorder that occurs primarily after the age of 65. The typical features are the impairment of memory, language, attention, executive functioning, apraxia, agnosia, and aphasia (*Lobo et al 2000; Alzheimer's Association et al 2011*). According to the amyloid hypothesis, AD is characterized by accumulation of senile plaques constituted by deposits of the abnormal form of amyloid protein ($Ab_{40–42}$ amino acids), present in common forms of dementia, and neurofibrillary tangles originating from hyperphosphorylation of microtubular tau protein.

However, today some different pathophysiological theories regarding AD exist, suggesting that the disease could be driven by inflammation, vascular changes, and metabolic disorders. These theories are not mutually exclusive, because inflammation plays a relevant role in both vascular lesions and metabolic disorders (*Vasto et al 2008; de la Torre 2004; Milionis et al 2008; Vasto et al 2007; Candore et al 2010a; Candore et al 2010b; Elks et al 2010*).

Indeed, several population-based studies have recently described MS and T2DM as a risk factors for AD. Furthermore, these data were also confirmed in the pre-AD status, the so-called mild cognitive impairment.

But from the age of 85 the association between MS and accelerated cognitive decline vanishes (*van den Berg et al 2007*). However, data are not

definitive and negative results have also been published (*Ahtiluoto et al 2010; Luchsinger 2010; Sanz et al 2009*).

In 2005, a new theory was proposed about the possibility to consider AD as a third form of diabetes. AD brains were analyzed postmortem, showing, in the frontal cortex, lower levels of insulin, IGF-1 and insulin receptor. These data showed that later stages of AD were associated with an up to 80% decrease of these parameters compared to healthy brain (*Steen et al 2005*). According to the latter association, some authors proposed the concept of ‘‘metabolic cognitive syndrome’’ when describing co-occurrence of AD and MS (*Frisardi et al 2010*).

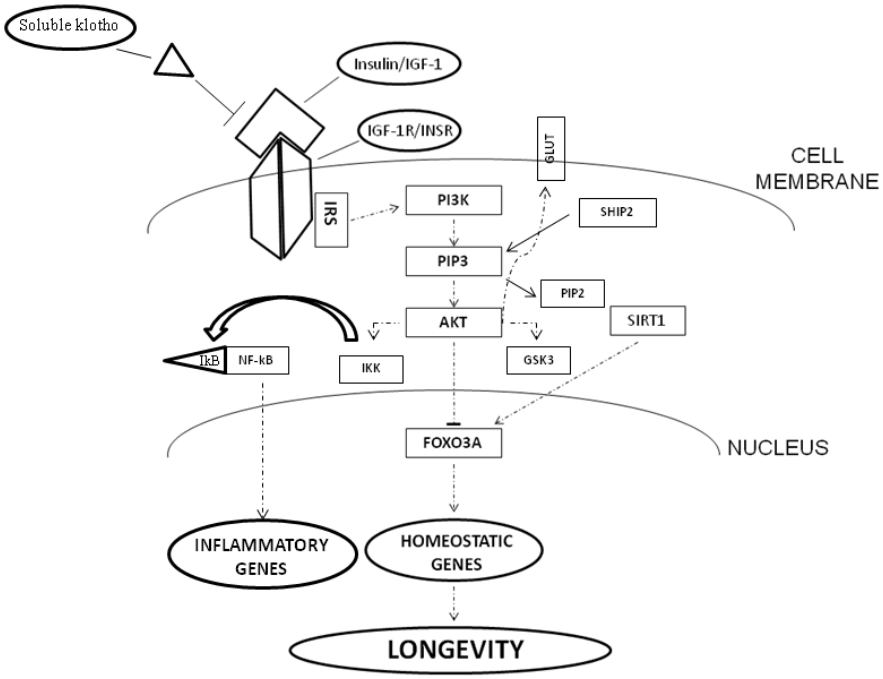
Environmental elements like diet, lifestyle, smoking, and socioeconomic status are critical contributors in these disorders and, from a molecular point of view, an impairment in insulin signaling pathway has been suggested to have a key role in their pathogenesis (*Wilcox 2005*).

Insulin is known to be a peripheral regulator of nutrient storage, but it is also essential for the control of energy balance and for many other functions (neurotransmitter release, neuronal outgrowth, tubulin activity, neuronal survival, and synaptic plasticity) in the central nervous system by processes not linked to modulation of glucose uptake that occurs in peripheral tissues (*Wang et al 1992; Tanaka et al 1995; Cole et al 2007*).

Neuronal insulin signaling pathway has an important function in mammalian fat storage and in *C. elegans* and *Drosophila*, the cellular signaling systems mediating these effects bear remarkable homology to those described in mammals (*Porte et al 2005*).

The pathways described are depicted in **Figure 4.**

Figure 4. INSULIN/IGF-1 PATHWAY AND AGEING, AGE-RELATED DISEASEAS AND LONGEVITY. When insulin (or IGF-1) binds to its receptor the IRSs contact the PI3K. PI3K activates the via of the second messenger PIP3. SHIP2 is an antagonist of PI3K and together regulate the levels of the second messenger PIP3: PI3K phosphorylates PIP2 giving PIP3; conversely SHIP2 removes a phosphate from PIP3 to obtain PIP2. This second messenger leads to the activation of AKT that activates IKK that inhibits IkB linked to NF-kB. Thus NF-kB can translocate to the nucleus acting as TF for inflammatory genes. Moreover, AKT inhibits FOXO3A preventing the transcription of homeostatic genes and acts on GSK3 too. Also SIRT1 can act on FOXO3A.
Outside the cell, a soluble form of KLOTHO can inhibit the insulin/IGF-1 signalin thus possibly contributing to the transcription of homeostatic genes and consequently to longevity.



1.4 DIFFERENT APPROACHES TO STUDY AGEING AND LONGEVITY

The ageing and longevity are multi-factorial events in which genetic, epigenetic, stochastic and environmental factors seem to have a crucial role (*Caruso et al 2012; Montesanto et al 2012*).

Approximately 25% of the overall variation in human lifespan 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% (*Herskind et al 1996; Ljungquist et al 1998; Skytthe et al 2003; Vaupel et al 1998*). Concerning the role of genetics, as we discussed in our paper (*Incalcaterra et al 2013*), three approaches are the most used and useful to assess biomarkers of ageing and longevity: the candidate gene approach, the meta-analysis and the genome-wide association studies (GWAS).

1.4.1 CANDIDATE GENE APPROACH

The candidate gene approach is a hypothesis-driven method widely employed by case–control studies. The case control study compares subjects with a specific disease or outcome of interest that represent the cases with subjects that do not have the disease or outcome that represent the controls.

The aim of this type of study is to analyzed if the different frequency of each event is manifested in a statistically significance manner, calculating the association with a chi square test to determine the relationship between the risk factor and the disease. It is an observational study because no interventions are made in the subjects analyzed. In particular, in our case,

the genotype and allele frequencies of two populations are compared: one affected and one unaffected by a complex trait. If the identified allelic variants are more prevalent in the affected population as compared to unaffected, these genotypes are associated with the trait. Of course, consistent replication of study in different population is needed to validate the data and to identify the variant as a biomarker.

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 particular gene, taking into account different gene-environment interactions.

In the last years, our group has extensively investigated by candidate gene approach the role of inflammation and in particular of specific immune-inflammatory molecules in ageing and longevity (*Balistreri et al 2012; Incalcaterra et al 2013; Balistreri et al 2013*). In the Table below (**Table 3**), extracted from an our recent report, are depicted the data obtained from the association of genetic variants with the risk of myocardial infarction (MI) and with longevity using centenarians as healthy controls.

These data are obtained in a Sicilian homogeneous population but are only partially agree with data previous shown in different population. In particular, the approach of the so called “positive biology” was used for this study. CO are characterized by a reduction in the onset of CVDs thus alleles associated with these diseases would not be included in the genetic background favoring longevity. The aim of “positive biology” is to focus the attention on the cause of positive phenotypes rather than pathologic to understand the biological mechanism of healthy ageing. The results in the table clearly demonstrate that in MI subjects, the SNPs investigated are overrepresented respect to centenarians that, conversely, present variants

that are protective against CVDs (*see the paper for the references, abstract n°3, n°5 and n°10*).

Table 3. GENETIC VARIANTS ASSOCIATED WITH MYOCARDIAL INFARCTION AND LONGEVITY.

Genes	Alleles of SNPs or genetic variants	Centenarians	Young controls (< 55 years)	MI patients(< 55 years)	*Pp* -value
TLR-4	+896A>G[1](Asp299Gly; rs4986790)	NN=55 males	N=127 males	N=105 males	< 0.001
CCR5	WT>Δ32[2](rs333)	N=123 males	N=136 males	N=133 males	=0.00006
Cox-2	−765G>C[3](rs20417)	N=96 males	N=170 males	N=140 males	=0.000007
5-LO	−1078G>A[4](rs2115819)21C>T	N=96 males	N=170 males	N=140 males	=0.00003
		N=96 males	N=170 males	N=140 males	=0.001
FLAP	−336G>A[5]	N=96 male	N=170 males	N=140 males	=0.0007
Cx37	−1019C>T[6]	N=56 males	N=196 males	N=97 males	=0.0035
IL-10	−1082G>A[7](rs1800896)	N=52 males	N=110 males	N=90 males	=0.0003
α1AT	342G>L [8]	N=143	N=255	N=127	=0.0000001
MEFV	694 M>V[9]	N=68	N=196	N=121	=0.003

[1] This missense polymorphism alters the extracellular domain of TLR-4, so it attenuates the TLR4 signaling pathway and diminishes the inflammatory response to Gram-negative pathogens. In particular, it may influence inflammatory responses and the risk of major inflammatory age-related diseases, such as AS, by affecting the production of inflammatory mediators.

[2] A non functional allele, resulting from a 32-bp deletion in exon 4, determines a loss of expression of functional CCR5 receptor. So, this genetic variant may have a protective role against AMI as consequence of an attenuated inflammatory response that should determine a slower progression of atherosclerotic lesion among CCR5Δ32 carriers.

[3] Located within a putative binding site for the transcription factor Sp1, associated with a different transcription of gene.

[4] SNPs in promoter region and exon-1 of 5-LO gene, respectively, able to modify the gene transcription or the putative protein.

[5] It has been claimed to be functional, modifying gene transcription or modifying the putative protein derived from gene translation.

[6] This SNP causes a shift from proline to serine at amino acid 319. In a mouse model of atherosclerosis, the mouse Cx37 protein was shown to be atheroprotective by properly regulating leukocyte recruitment, namely one of the first inflammatory steps in atherosclerotic process.

[7] SNP in the IL-10 proximal gene region (considered potential target for transcription regulating factors) involved in genetic control of IL-10
production, even if contrasting literature data have been reported. In particular, the homozygous -1082GG genotype seems to be associated with higher IL-10 production

respect to G>A heterozygous and AA homozygous genotypes. Furthermore, this SNP seems to be functionally relevant. It has been demonstrated that -1082 A carriers (low producers) seem likely develop a major number of chronic inflammatory diseases.

[8] It results in a severe protein deficiency that is characterized in the homozygote state by levels of plasma concentrations that are lower by 84 % when compared with levels in MM individuals and in the MZ heterozygote state, by intermediate levels that are lower by 17 %. It has been suggested that α1AT deficiency could lead to less cleaved fragments of α1AT (i.e. the pro-inflammatory peptide C-36) of α1AT in atherosclerotic plaques, and thereby reduce AS inflammatory process..

[9] This mutation in the pyrin gene is liable to lead to leukocyte survival otherwise designed to follow the apoptotic pathway, increasing the
inflammatory response.

Reference in *Incalcaterra et al 2013, abstract n°5.*

1.4.2 GENOME-WIDE ASSOCIATION STUDY

As we discussed in our paper (*Incalcaterra et al 2013*), GWAS consists in a scanning of whole genome, analyzing 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 to analyze.

Population admixture that may produce possible false positives due to different genetic backgrounds among ethnic groups is another limit. A family-based association study and the analysis of geographically isolated population could permit to improve the detection of true positive genetic association loci, in particular those of modest size (*see paper for the references, abstract n°5*).

GWAS is a useful tool for the identification of longevity associated alleles with frequency above 5%. Each, taken singularly, has a moderate or null effect but, borrowing a concept from pharmacology, it is possible to

speculate that more alleles have, en bloc, a synergic rather than an additional effect. It means a potentiated effect respect to the sum of alleles. On the other hand, GWAS is not the best tool to study longevity because it is necessary to collect thousands of individuals to identify the expected OR.

For this reason it could be preferred to use GWAS to formulate an hypothesis verifying it with different traditional research methods.

In this aspect, a GWAS on 410 long lived individuals (LLI) and 553 young control individuals investigated the relation between rs10491334 of the calcium/calmodulin-dependent protein kinase IV (CAMKIV) gene, a variant previously reported in association with diastolic blood pressure, and human longevity. To confirm this association *in vitro* study was conducted establishing that CAMKIV activates the survival proteins AKT, SIRT1, and FOXO3A, thus resulting indirectly involved in insulin pathway. Moreover, homozygous carriers of rs10491334 have a significant reduction in CAMKIV expression pointing to a detrimental role for the SNP and to its involvement with human longevity (*Malovini et al 2011*).

1.4.3 SYSTEMATIC REVIEW AND META-ANALYSIS

Systematic review and meta-analysis are two strictly linked very useful tools for the researchers. In particular, a systematic review permits to explore the existing literature to verify if data of different studies in different populations about the same variable have a significance if they are analyzed all together. It typically involves several steps: the identification of the question, the selection of databases, the choose of the adapt strategy, the management of the filters in the database to select paper that answer the original question (inclusion and exclusion criteria) and the extraction of the

data in a standardized format. A "meta-analysis" provides a statistic tool to quantitatively synthesize and combine the data selected with the systematic review. Thus “every meta-analysis should be based on an underlying systematic review, but not every systematic review leads to a meta-analysis” (*http://researchcore.org/faq/answers.php?recID=5*).

Thus, the use of meta-analyses has recently become an important part of genetic research mainly to reconcile studies about the same genetic variant that gave inconsistent results.

One interesting “pro” of these is for example the possibility to overcome the limit of GWAS linking to the standard threshold of significance, highlighting genes or gene variants with weak effect on a specific phenotype as associated to that phenotype. This is particularly relevant in longevity research because it is well known that genetic background and longevity are strictly linked but few genetic variants associated with longevity exist.

With these approaches, besides our results on the association of specific allelic variants of IGF-1R, FOXO3A with longevity and KLOTHO with health ageing, many other genetic variant were pointed out to ageing and longevity research for a gain or a loss of association: the negative association with longevity in Italian centenarians of the GG genotype of interleukin-6 previous associated in European centenarians (*Di Bona et al 2009*) or the positive association with longevity of a specific haplotype (rs915179, rs2485662, rs4641, rs1468772) from LMNA gene (*Conneely et al 2012*).

Moreover, putting together similar genetic data of 5 studies of centenarians from USA, Europe and Japan, the result was that many of the variants analyzed in these studies were associated to extremely long life

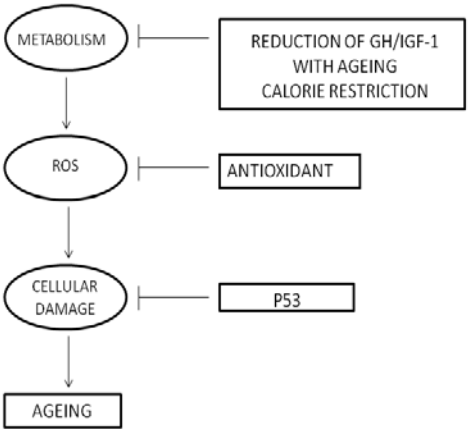
(*Sebastiani et al 2013*). However, a weakness of meta-analysis is that it does not overcome problems that were inherent in the design and execution of primary studies.

Combining studies of poor quality with those that were more rigorously conducted is not useful and can lead to worse estimates of the real effect.

2. AIM OF THE THESIS

The new frontier in ageing investigation is the promotion of healthy ageing and longevity, rather than the intervention on specific age-related diseases. In a recent consensus workshop held in Erice, it has been talk about possible interventions to slow ageing (*http://longevityinstitute.usc.edu/SicilyMeetingProgramFINAL2013.pdf*) (**Figure 5**).

Figure 5. MECHANISMS THAT DELAY AGEING PROCESS. The main source of damage for the macromolecules, including the DNA, is the metabolism that determines over production of radical oxygen species (ROS) due to the reduction of efficiency of the mithocondria. Low levels of GH/IGF-1 likely increase the efficiency of mitochondria with a consequent reduction of ROS production. At the same time, the low levels mean prevention of insulin resistance and consequently control of inflammation process. Limited levels of ROS are also due to antioxidant mechanism physiologically active inside the cells. P53 is usually inactive because of is binding with MDM2. Stress and environmental stimuli determine the activation of p53 that has the role to maintain genomic stability. All these systems contribute to delay ageing process.



Hence, the new goal is to increase lifespan but in healthy condition: the “heal thspan”. It is well known that reduction of nutrient sensing pathway signaling increases lifespan in model organisms.

In rodents, calorie restriction without malnutrition, reduces insulin/IGF-1 signalling, 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*).

However, since calorie restriction is difficult to realize in human beings, it may be preferred to reach healthy ageing acting on modifiable lifestyle factors as diet and nutrition. Moreover, further target should be the control of pro-inflammatory status, characteristic of ageing, as extensively discussed in our reports (*Balistreri et al 2013, abstract n°12*; *Accardi et al 2014; Balistreri et al 2014*).

Thus the aim of my PhD was to explore the mechanisms that drive ageing and longevity focusing the attention on the role of insulin/IGF-1 pathway and of inflammatory mechanisms, well known to be among the main driver to these phenotypes. Through reviews we explored the literature to summarize the existing data up to date. With systematic reviews and meta-analyses we identified some genetic variants of proteins that take part in the insulin/IGF-1 pathway, both directly or indirectly. Moreover, we conducted a case-control study to verify the association of two SNPs of the protein SHIP2 to T2DM and AD.

The obtained results will be discussed in the light of the methods to slow ageing.

3. Can Alzheimer Disease Be a Form of Type 3 Diabetes?

REJUVENATION RESEARCH
Volume 15, Number 2, 2012

DOI: 10.1089/rej.2011.1289

Can Alzheimer Disease Be a Form of Type 3 Diabetes?

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Abstract

Alzheimer disease (AD) and metabolic syndrome are two highly prevalent pathological conditions of Western society due to incorrect diet, lifestyle, and vascular risk factors. Recent data have suggested metabolic syndrome as an independent risk factor for AD and pre-AD syndrome. Furthermore, biological plausibility for this relationship has been framed within the "metabolic cognitive syndrome" concept. Due to the increasing aging of populations, prevalence of AD in Western industrialized countries will rise in the near future. Thus, new knowledge in the area of molecular biology and epigenetics will probably help to make an early molecular diagnosis of dementia. An association between metabolic syndrome and specific single-nucleotide polylmorphisms (SNPs) in the gene INPPL1, encoding for SHIP2, a SH2 domain-containing inositol 5-phosphatase involved in insulin signaling, has been described. According to recent data suggesting that Type 2 diabetes represents an independent risk factor for AD and pre-AD, preliminary results of a case–control study performed to test the putative association between three SNPs in the SHIP2 gene and AD show a trend toward association of these SNPs with AD.

Introduction

Alzheimer disease (AD) is the most common form of dementia, accounting for more than 50% of all cases of dementia.[1] It occurs primarily after age 65, and for this reason it is classified as an age-related disease. The exception is the familiar early-onset form (with Mendelian inheritance) that represents about 1% of all cases.[2] Its prevalence is approximately 1% between 65 and 69 years and is higher than 50% in individuals above 95 years.[3] AD is a neurodegenerative disorder with the typical features characterized by the impairment of memory, language, attention, executive functioning, apraxia, agnosia, and aphasia. Cognitive, but also behavioral, symptoms cause a reduction of functional activities compared to a previous level of functioning.[1–3]

According to the amyloid hypothesis, AD is characterized by accumulation of senile plaques constituted by deposits of the abnormal form of amyloid β (Aβ) protein (A$\beta_{40–42}$ amino acids), present in common forms of dementia, and neurofibrillary tangles originating from hyperphosphorylation of microtubular tau protein. These structures accumulate progressively in the brain starting from the hippocampus and then spreading to the cerebral cortex, where neurons are lost, causing memory, language, and general cognitive impairment.[3]

However, today some different pathophysiological theories regarding AD exist, suggesting that the disease could be driven by inflammation, vascular changes, and metabolic disorders. These theories are not mutually exclusive, because inflammation plays a relevant role in both vascular lesions and metabolic disorders.[3–8] Indeed, several population-based studies have recently described Type 2 diabetes as a risk factor for AD. Furthermore, these data were also confirmed in the pre-AD status, the so-called mild cognitive impairment. However, data are not definitive and negative results have also been published.[9–11]

Most recently, metabolic syndrome, which represents a cluster of metabolic factors—insulin resistance, abdominal obesity, glucose intolerance, hypertension, hyperinsulinemia, and raised fasting plasma glucose—has also been described in association with an increased risk of AD.[12,13] Interestingly, strong evidence suggests that systemic inflammation and central adiposity contribute to and perpetuate metabolic syndrome.[8] All of these alterations predispose individuals to type 2 diabetes and cardiovascular disease.[8–14]

Genetic background, age, sex, diet, physical activity, and habits in general all influence the prevalence of the metabolic syndrome and its components. Twenty years ago in the Mediterranean area, it was assessed that 70% of adults have at least one of the disorders characterizing metabolic syndrome. However, in the European population, the rate

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of metabolic syndrome is 7%–30%.[15,16] Worldwide there are 1.1 billion overweight people with a body mass index (BMI) between 25 kg/m^2 and 30 kg/m^2 and 312 million with a BMI >30 kg/m.[14] In the last 40 years, the rate of obesity in the United States has increased, and today 66% of adults have a BMI >25 kg/m^2 and half of those have a BMI >30 kg/m.[17]

Another link between obesity, inflammation, insulin signaling, and dementia is the amyloid precursor protein (APP),[18] a transmembrane protein from which the A$\beta_{40–42}$ fragment that forms senile plaques originates.[3] APP is considered an adipokyne, producing and processing A$\beta_{40–42}$ in adipose tissue. This fragment is expressed in fat tissues and overexpressed in abdominal adipocytes of obese patients.[18]

Recent data support an increased susceptibility for AD in patients with metabolic syndrome,[19] but from the age of 85 the association between metabolic syndrome and accelerated cognitive decline vanishes.[20] On the other hand, some American scientists hypothesize that AD is a third form of diabetes.[21] This hypothesis was formulated in 2005 when 45 AD patients were analyzed postmortem, showing lower levels of insulin in the brain. In particular, the authors analyzed the frontal cortex of AD individuals, calculating the concentration of insulin, insulin-like growth factor 1, and insulin receptor. Data showed that later stages of disease were associated with an up to 80% decrease of these parameters compared to healthy brain.[21] According to the latter association, some authors proposed the concept of "metabolic cognitive syndrome" (MCS) when describing co-occurrence of AD and metabolic syndrome. Indeed, dementia and metabolic syndrome present some overlap both in predisposition factors and in altered signaling cascade. Environmental elements like diet, lifestyle, smoking, and socioeconomic status are critical contributors in these disorders. Altered insulin signaling pathway has a key role in their pathogenesis. In particular insulin resistance might be the first step toward both disorders, constituting a bridge between AD and metabolic syndrome.[22]

Metabolic-Cognitive Syndrome: Insulin and the Central Nervous System

Insulin is known to be a peripheral regulator of nutrient storage, but it is also essential for the control of energy balance in the central nervous system (CNS). Neuronal insulin signaling pathway has an important function in mammalian fat storage and in *Caenorhabditis elegans* and *Drosophila*, the cellular signaling systems mediating these effects bear remarkable homology to those described in mammals.[23]

There is substantial evidence demonstrating insulin action in the control of neuronal function in cortical and hippocampal areas, which are involved in memory processing and cognitive functioning.[24,25] Insulin directly influences neurons by processes not linked to modulation of glucose uptake. Neurotransmitter release, neuronal outgrowth, tubulin activity, neuronal survival, and synaptic plasticity are all directly modulated by insulin.[26–29] The insulin signaling pathway modulates synaptic plasticity, promoting the recruitment of γ-aminobutyric acid (GABA) receptors on postsynaptic membranes, influencing *N*-methyl D-aspartate receptor (NMDA) conductance (neuronal $Ca2^+$ influx) and regulating receptor α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) cycling.[30,31]

The MCS was elaborated on 2010 by Frisardi and his colleagues. It is based on the co-existence, in patients, of metabolic syndrome and cognitive impairment of degenerative or vascular origin.[22] Insulin resistance can be manifested in peripheral tissues or directly in the brain as an insulin resistance brain state that contributes to cognitive impairment and neurodegeneration for the reason described above.[22]

Many molecules participate in the regulation of the insulin signaling pathway; therefore, an alteration in the function or expression of some of these proteins causes a reduction in glucose uptake. Consequently, glucose accumulates in the blood, determining hyperglycemia and hyperinsulinemia. Hyperglycemia induces an increase of the peripheral use of insulin, which results in a reduction of insulin disposable for the brain. Because insulin is essential for memory, learning, neuronal survivor, and longevity processes, the alteration of its concentration might cause important consequences on tau and Aβ processing.[24,25] For example, an impairment of insulin signaling pathway causes a reduction of the activity of phosphatidylinositol 3-kinase (PI3K) and consequently a reduction in AKT/protein kinase B (PKB) pathway. This leads to an increase of glycogen synthase kinase 3 α/β (GSK-3 α/β) activity that phosphorylates tau protein and causes intraneuronal Aβ accumulation.[21]

Moreover, glucose metabolism plays a role in the protein posttranslational modification involving the hexosamine biosynthetic pathway, which leads to the generation of *O*-*N*-acetylglycosamine (O-Glc-NAc). If insulin resistance is established, intraneuronal glucose metabolism is impaired. Consequently, the amount of O-Glc-NAcylation is reduced. This posttranslational modification competes with the phosphorylation process, thus more phosphate groups are added with an increase of the amount of phosphorylated tau protein.[32]

Insulin is also involved in the APP metabolism.[33] APP competes with the insulin receptor. Thus, its inefficient degradation might play a key role in AD brain insulin resistance.[34]

SHIP2: A Modulator of the Insulin Pathway

When insulin binds to its membrane receptor, it activates a signaling cascade involving phosphoinositides and the AKT/PKB pathway.[24] To regulate cellular levels of lipid secondary messengers such as phosphatidylinositol (3,4,5)-triphosphate (PtdIns [3,4,5]P3), cells use two major classes of phosphoinositide phosphatases—the inositol polyphosphate 3-phosphatase PTEN and the SH2 domain-containing inositol 5-phosphatases 1 and 2 (SHIP1 and SHIP2).[35]

SHIP2 is a protein that catalyzes the degradation of lipid secondary messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) to produce phosphatidylinositol 3,4-diphosphate (PIP2). Thus, SHIP2 is an antagonist of PI3K that takes part in insulin signaling, phosphorylating PIP2 to obtain PIP3. Because the PI3K pathway plays a key role in the biological effects of insulin, the attenuation of the PI3K-mediated insulin signaling pathway could be associated with insulin resistance in type 2 diabetes.[36]

Many studies underline the role of SHIP2 as negative regulator of insulin signaling.[35–37] Its overexpression reduces both insulin-stimulated mitogen-activated protein kinase and AKT activation, leading to downregulation of glucose uptake toward failed recruitment of GLUT4 in cell membrane and glycogen synthesis in 3T3-L1 adipocytes and L6 myotubes.[38–40] Moreover expression of SHIP2 is greatly increased in the skeletal muscle and fat tissue of diabetic mice.[41]

In addition, the SHIP2 gene (INPPL1) is localized in human chromosome 11q13–14, which is suggested to be linked to type 2 diabetes characterized by insulin resistance and hypertension.[42–44] Therefore, SHIP2 could be involved in the pathogenesis of insulin resistance of type 2 diabetes mellitus in humans and also in the metabolic syndrome, in which insulin resistance represents the first step toward.[36,41–43,45]

A study conducted by Kaisaki et al.[45] shows a significant association between single-nucleotide polymorphisms (SNPs) of INPPL1 (rs2276047, rs9886, and an insertion/deletion in intron 1) and type 2 diabetes and metabolic syndrome in European populations. This finding was partly confirmed by another study conducted by Kagawa et al. in the Japanese population.[46]

Conclusion

Metabolic syndrome and AD constitute a worldwide problem, especially for Western societies, due to co-morbidity (mainly vascular), lifestyle (*i.e.*, diet, exercise, smoking, alcohol), and increasing age. Considering the increasing data that have focused recently on the association between AD and metabolic syndrome, it could be speculated that AD could be a third form of diabetes.[21]

Metabolic syndrome is a condition that predisposes to type 2 diabetes, which is characterized by systemic inflammation, insulin resistance, obesity, high cholesterol levels, and sedentary lifestyle, all conditions related to an increased risk for AD.[47] Due to increasing age, prevalence of AD in Western industrialized populations will be higher in the future. Thus, new knowledge regarding molecular biology and epigenetics that would enable an early molecular diagnosis of dementia is welcome.[3]

Discovery of new genes and proteins involved in physiological pathways can be crucial for the identification of altered mechanisms involved in the pathophysiology of AD and consequently in signaling pathways. Such discoveries would allow finding new target proteins, developing new molecular risk profile for diagnosis and prevention, and planning early interventions.[6] In this regard, we are extending previous research on the association of INPPL1 SNPs and metabolic syndrome to AD. With this aim, we are conducting a case–control study evaluating the putative association between INPPL1 SNPs and AD. Preliminary results obtained show a trend toward association of these SNPs with AD, thus strengthening the hypothesis of a close relationship among AD, metabolic syndrome, and diabetes.

Acknowledgments

Original work discussed in this review was supported by grants from the Ministry of Education, University, and Research ex 60% to C.C. G.A. is a Ph.D. student of the pathobiology Ph.D. course (directed by C.C.) at Palermo University, and this paper is submitted in partial fulfillment of the requirement for her Ph.D. degree.

Author Disclosure Statement

No competing financial interests exist.

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4. NF-κB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies

IMMUNITY & AGEING

REVIEW Open Access

NF-κB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies

Carmela R Balistreri*, Giuseppina Candore, Giulia Accardi, Giuseppina Colonna-Romano and Domenico Lio

Abstract

Chronic inflammation is a major biological mechanism underpinning biological ageing process and age-related diseases. Inflammation is also the key response of host defense against pathogens and tissue injury. Current opinion sustains that during evolution the host defense and ageing process have become linked together. Thus, the large array of defense factors and mechanisms linked to the NF-κB system seem to be involved in ageing process. This concept leads us in proposing inductors of NF-κB signaling pathway as potential ageing biomarkers. On the other hand, ageing biomarkers, represented by biological indicators and selected through apposite criteria, should help to characterize biological age and, since age is a major risk factor in many degenerative diseases, could be subsequently used to identify individuals at high risk of developing age-associated diseases or disabilities. In this report, some inflammatory biomarkers will be discussed for a better understanding of the concept of biological ageing, providing ideas on eventual working hypothesis about potential targets for the development of new therapeutic strategies and improving, as consequence, the quality of life of elderly population.

Keywords: Biological ageing process, Inflammatory network and its effects in ageing, NF-κB signaling pathway as hub of inflammatory ageing network, Inflammatory biomarkers

Introduction

Ageing is a complex process, induced by an intricate interaction of genetic, epigenetic, stochastic and environmental factors. They determine the lost of molecular fidelity followed by an improved entropy [1,2]. As result, loss complexity and random accumulation of damages (i.e. particularly damages to nuclear and mitochondrial DNA) at cellular, tissue, organ levels and/or of whole body arise, compatibly with the disposable soma theory of ageing [3]. Thus, it establishes a condition, which modifies both architecture and functioning of physiological processes and regulatory (*immune and endocrine*) systems. This determines a deterioration of the homeostasis. Accordingly, it becomes more easily vulnerable to internal and external stressors, frailty, disability and disease (Figure 1). On the other hand, the loss of DNA integrity, the principal random damages able in modifying cellular fidelity and inducing cellular and whole body senescence, determines the decline of the functionality of stress resistance and survival pathways (i.e. autophagic uptake mechanisms, chaperone systems, DNA repair mechanisms, apoptotic process, immune/inflammatory response), involved in cellular and organism defense to environmental stress and maintaining homeostasis [2]. However, a large heterogeneity in occurrence, complications, speed, and age and gender manifestation of ageing process at cellular, tissue, organ levels and/or of whole body has been observed in humans. Among human people, there are individuals at the age ≥90 years still in good mental and physical conditions, and others that at the age ≥ 60 years show cognitive difficulties, and/or the onset of chronic inflammatory diseases, such as Alzheimer's disease (AD), cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) and cancer [4].

The principal causes of the heterogeneity in human ageing rate, measured as decline of functional capacity and stress resistance, seem to be genetic and environmental factors. However, the overall impression is that environmental factors are the major determinants of both ageing and age-related diseases [4,5]. Thus, ageing process is not a genetically programmed process [5]. This consideration is based on studies on heritability of age-related diseases and ageing [6,7]. A similar value of heritability in lifespan and age-related diseases, such as

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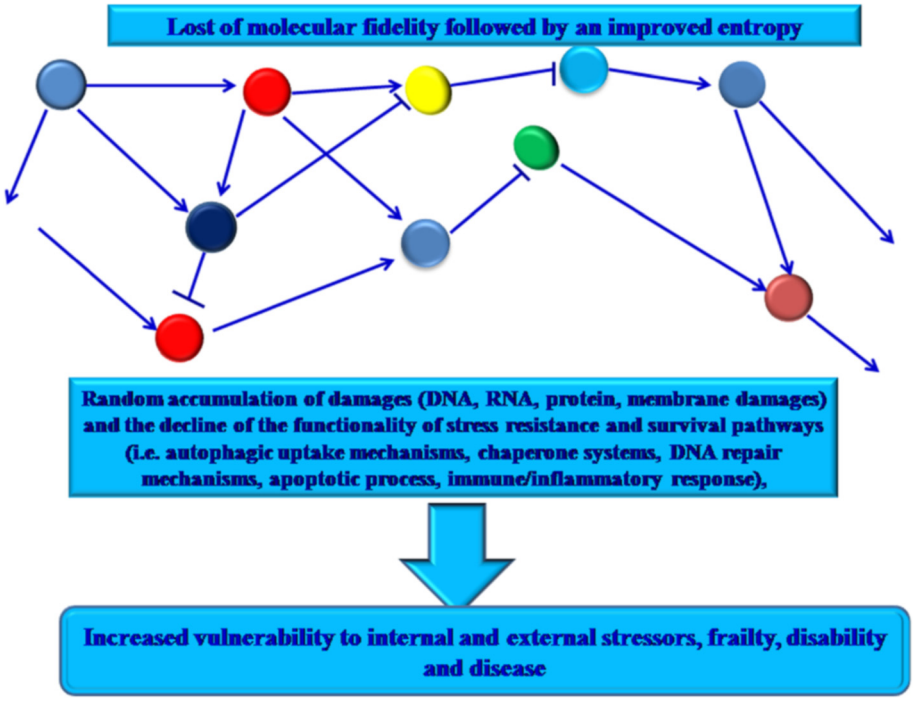

Figure 1 Ageing process results by the lost of molecular fidelity followed by an improved entropy. This determines loss complexity and random accumulation of damages (i.e. particularly damages to nuclear and mitochondrial DNA) at cellular, tissue, organ levels and/or of whole body. Thus, it becomes more easily vulnerable to internal and external stressors, frailty, disability and disease.

cancer, AD, CVD and T2DM, has been identified (35% *vs*. 40%, respectively) [8,9]. However, this does not imply that genetic factors have an irrelevant role in ageing and age-related diseases. For example, mutations identified in familial forms of AD consented understanding its molecular mechanisms, such as the toxicity of amyloid β peptide and potential therapeutic targets in more common sporadic late onset AD [10]. Common suggestion is based on both a complex contribution of genetic factors in ageing and diseases of later life and weak effects of individual genes [5]. Furthermore, diverse genetic factors are associated with ageing and exceptional longevity. Human genome-wide genetic analyses have revealed only few age-related loci and polymorphic longevity genes [11-13]. Among these, current promising candidates are Sirtuins, Forkhead box O protein (FoxOs) and the field of epigenetics. Functional genomics, i.e. expression profiling studies, have revealed a group of genes which are differently expressed in ageing, such as immune/inflammatory genes [14].

From the observations described above, another critical point of ageing process emerges based on the concept of biological age as real expression in human of both ageing rate and onset of the common diseases of later life rather than chronological age [15]. This concept opened an important area of research focused on identifying of potential molecular targets as *biomarkers of human biological ageing* [16]. On the one hand, it could consent to develop potential anti-ageing treatment strategies. On the other hand, probable anti-ageing treatments could retard or prevent age-associated diseases resulting in widespread health, social and economic benefit. Such treatment could include genetic engineering, such as gene therapy or endogenous gene repair, or pharmacological therapies, or changes in lifestyle, i.e. physical activity, diet.

In this report, many of these aspects are discussed, giving particular emphasis in describing some biomarkers of inflammation. In particular, the data discussed in this report are based on an expert opinion derived on the findings from author's studies on ageing, age-related diseases and inflammation.

Definition and selection criteria of ageing biomarkers

As established by National Institute of Health, a biomarker is a "*feature objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [16,17].

In the case of ageing process, this definition might concern measures related to physical changes, such as grey hairs, reduced skin elasticity, wrinkles, reduced muscle strength or changes in the near vision, which are thought to be the result of molecular mechanisms

occurring in the old age [18]. However, they reflect the chronological age rather than the biological age, which is the most important indicator of health and potential lifespan [15].

A biomarker of real ageing should reflect a process of biological ageing, be easily reproducible in cross-species comparison, be easily obtainable. Since ageing is the result of the deterioration of more than one system or process, it would be more appropriate to consider different biomarkers. Panels of biomarkers associated with conditions, alterations or changes of a set of critical systems to assess the biological age of any organism should be used [16,18].

The gerontologists have begun to face this problem already in the early 1980s, with the development of a large number of ageing biomarkers [16,18]. Despite the numerous efforts and the support in this research from National Institute of Ageing, the major number of biomarkers is still to date under discussion, like inflammatory markers, hormones, markers of oxidative stress or telomere shortening [16,18]. Most (perhaps all) markers are also not really proven in longitudinal studies in humans. In addition, they have been considered for a variety of purposes, which are not distinguished sufficiently. Most studies used biomarkers as tools for comparing ageing rate in several populations or cohorts of a single population. In contrast, others considered biomarkers for person-specific predisposition, which represents much more challenging, principally because ageing, as a biological process, is not well defined at individual level. In addition, the research of comparative or predictive biomarkers have determined the attempted use of measure panels associated with survival, health of old age, frailty, age-related (multi) morbidity and mortality [16,18].

However, none of the identified biomarkers is a "*real*" biomarkers of ageing. They are commonly related to age and diseases. In addition, the major number has been developed and tested for diseases in which biological age is the single biggest risk factor, such as peripheral blood cellular telomere length, indicators of immunosenescence, even without correlations with disease-specific diagnoses. In addition, biomarkers of age-related diseases and ageing have been preferentially identified in younger-old populations (typically aged 60–85), but not in oldest-old (aged 85 and above) [16,18]. For example, blood pressure, indicators of metabolic syndrome and telomere length do not associate significantly with age-related morbidity or mortality in population-based studies of the oldest-old [19-21]. Thus, biomarkers of ageing and age-related diseases in understanding the health trajectories of the oldest-old are unexplored. It is important that this lacuna is filled given the rapid growth in the number of very old people in many contemporary populations.

For an ageing biomarker, it is important to know not only its definition, but also the criteria for its selection. Accordingly, the American Federation for Ageing Research suggested detailed criteria, recently reviewed by Sprott [16] and Johnson [18]. Based on these criteria, a true biomarker of ageing, in order to be both accurate and useful, should predict a person's physiological, cognitive and physical function in an age-related way. In the same time, it should be easily testable, and not harmful to test individuals. For example, it could be a blood test or an image technique, by performing accurately and reproducibly without the need for specialized equipment or techniques. It should be tested preliminary in laboratory animals, such as mice, and successively in humans. Thus, a biomarker needs to be simple and inexpensive to use. They should cause little or no pain and stress (see Table 1) [16,18].

Furthermore, current research on ageing biomarkers is also focusing in identifying molecules which also are able in giving clinical indications. On the other hand, biomarkers represent a hot topic and have the ability to change our life, if real prediction, on an individual basis, can be made in the future.

Description and ageing biological effects of inflammatory network

Immune system is evolved to defend the host against microbial invasion, and to counteract tissue damage elicited by chemical or physical agents or trauma, maintaining consequently the homeostasis and tissue repair [22]. In both conditions, it responds in a appropriate manner by inducing apposite reactions (i.e. of suitable degree, with the involvement of a different array of cells and mediators), called *inflammatory responses* [22,23]. Inflammatory responses can be evocated initially

Table 1 Criteria for a biomarker of ageing process

I	It must predict the rate of ageing. In other words, it would estimate where a person is in their total lifespan. Operationally, it must be a better predictor of lifespan than chronological age alone.
II	It must monitor a basic process that underlines the ageing process, not the effects of diseases.
III	It must be able to be tested repeatedly without harming the person, for example, a blood test or an imaging techniques.
IV	It must be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in laboratory animals before being validated in humans.

as localized tissue reactions and subsequently as *acute phase reaction*, represented by systemic cytokine-induced reactions, including leukocytosis, fever, somnolence, anorexia, activation of hypothalamic-pituitary-adrenal axis and increased level of glucocorticoids, and acute phase synthesis, i.e. C reactive protein (CRP), in the liver. A complex network of molecules (the *mediators*) and cells (*neutrophils, monocytes, mast cells, endothelial cells, etc.*) characterize these reactions. They work together in concert and interact mediating the activation of different signaling pathways and the expression and transcriptional regulation of hub genes. The hub genes receive and direct the activity of many other genes [22,23]. Thus, these responses are induced through an inflammatory network. Recent studies on topology of this network evidence the crucial role of some mediators in driving the different cellular interactions and regulating the type of inflammatory reaction. Several mediators as pro- and anti-inflammatory molecules are involved [24]. Their release is modulated by different factors linked to well-known Nuclear Factor (NF)-κB pathway [25,26]. In addition, the magnitude of their production varies individually because of genetic heterogeneity. Single nucleotide polymorphisms (SNPs) in several genes and epigenetic factors seem to be involved [27]. Among the inflammatory mediators, the classical pro-inflammatory cytokines, Tumor necrosis factor α (TNF-α), Interleukin-1 (IL-1) and IL-6, play a key role. They are able in inducing both local and systemic effects [28]. When the causes of the inflammatory reaction are of a high intensity, their production is increased. Thus, they are released in the circulation provoking the acute phase response. In contrast, the anti-inflammatory cytokines, such as IL-10 are able to regulate the activation of inflammatory cells, by inhibiting the release of pro-inflammatory cytokines and therefore turning off the inflammatory processes [29].

Whether tissue health is not restored or in response to stable low grade irritation, inflammation becomes a chronic condition provoking continuous damages in the surrounding tissues. The collateral damage caused by this type of inflammation usually accumulates slowly, sometimes asymptomatically for years but can eventually lead to severe tissue deterioration [30].

From the above, it emerges that the inflammatory response is not *per se* a negative phenomenon. It is programmed by the evolution in neutralizing infectious agents, playing a beneficial role until the time of reproduction and parental care. In contrast, in old age, in a period largely not foreseen by evolution, it can determine a detrimental effect through chronic inflammatory responses ("antagonistic pleiotropy") in several/ all tissue and organs, which are cause of both the ageing phenotype and chronic diseases [24,30]. A low chronic grade of inflammation, the *"inflammageing"*, characterized by a 2 to 4-fold increase in serum levels of inflammatory mediators has been identified in ageing [31]. It seems to be as optimal predictor of mortality and, as mentioned above, a critical risk factor in the pathogenesis of several age-related chronic diseases as AD, CVD, T2DM, sarcopenia, frailty and functional disability [32].

Augment of age-related body fat and consequent increase of visceral adiposity, age-related decline of sex hormones, oxidative and genotoxic stress, cellular and tissue damage, nutrition, alterations of physical condition of gut microbiota, other organs (brain, liver) and systems (immune and endocrine) have been associated with inflamm-ageing [32-34]. In addition, factors linking to physiological stress, such a long-term smoking and depression, seem also to contribute to inflammageing [32-34]. However, the most important factor for age-related inflammation is the long-life pathogen burden [30]. Some recent studies have, indeed, evidenced associations between past infections and levels of chronic inflammation and increased risk of heart attack, stroke, and cancer [32,34]. For instance, persistent peripheral multibacteria infection, such as periodontitis, associated with gram-negative anaerobic bacteria capable of exhibiting localized and systemic infections in the host, is considered as possible aggravating cofactor in subjects with vascular diseases and risk factor for the onset of other age-related diseases, such as AD [30].

Of special relevance is the inflammation status in centenarian people. The literature data seem to be apparently contradictory. Increased levels of both inflammatory and anti-inflammatory mediators and significant frequencies of protective genotypes have been assessed in centenarians than the old subjects [30,35]. As consequence, identifying of apposite biomarkers likely in long lived subjects should be necessary. This might permit a preferential and selected development of pleiotropic therapeutic interventions acting concomitantly on different targets and at different levels.

Inflammatory ageing biomarkers: the crucial role of NF-κB activators

Ageing is not a genetically programmed process, as described above [5]. In contrast, it is recognized as an entropic process, characterized by loss of molecular fidelity and subsequent accumulation of different products [1,2]. In addition, it has been recently proposed that during evolution the host defense and the ageing process have become linked together [2]. Host defense and ageing mechanisms seem to be overlapping. In particular, host defenses seem to be involved in ageing process, to active inflammatory network and also to evocate the release of so-called *senescence associated secretory phenotype* (SASP), represented by a myriad of factors, such as the pro-inflammatory mediators [36,37]. A large range of

defense factors and mechanisms are involved in inducing of inflammatory network, and are all (or the major number) linked to the NF-κB pathway, an ancient signaling pathway specialized to the host defense [25,26]. In particular, the NF-κB system is a cytoplasmatic sensor constituted by a protein-complex (Rel family proteins-RelA/p65, c-Rel and RelB- and NF-κB components-p50/p105 and p52/p100) and inhibited commonly by binding to IκB proteins (IκBα, IκBβ, IκBγ, IκBδ, IκBε, IκBζ and Bcl3). In some cases, its inhibition is induced through the action of several signaling pathways and negative feedback loops acting through different mechanisms at various levels of signaling cascades. In contrast, its activation can be evocated both by immune insults and external and internal danger signals associated with senescence and ageing process, such as oxidative and genotoxic stress and tissue injuries [25]. Namely, its induction is linked to several recognition pathway, i.e. Toll-like receptors (TLRs) and inflammasome, as well as through different upstream kinase cascades via canonical or non-canonical pathways. IKKα/β and NIK are the most important upstream kinases, although several kinases can directly regulate the transcriptional capacity of NF-κB factors. IKKγ, generally called NEMO, is an important regulatory component of the IKK complex being linked upstream to genotoxic signals and IL-1 and TNF receptor mediated signaling. Activating kinases phosphorylate IκB proteins which are released from the complex and then degraded in proteasomes. Subsequently, the NF-κB complexes, having the crucial role of pleiotropic mediator of gene expression, translocate into the nucleus and transactivate the expression of special sets of target genes, codifying different SASP molecules, including pro-inflammatory cytokines, chemokines, adhesion molecules, eicosanoids, growth factors, metallo-proteinases, nitric oxide, etc. [25]. On the other hand, emerging experimental data principally performed on human skin fibroblasts have convincingly demonstrated the role of NF-κB signaling as the major pathway stimulating SASP phenotype [36-40]. Among the endogenous NF-κB inducers, a particular action is mediated by oxidative stress, DNA damage and immune defense, which are typical features of the entropic ageing process and age-related diseases [25].

These observations lead in considering NF-κB as hub of ageing inflammatory network, whose the mentioned factors act as NF-κB activators and pro-ageing factors. With advancing age, these factors increase and determine a sustained NF-κB activation, eliciting a host defense "*catastrophe*", responsible of SASP release. In turn, SASP, which occurs in several cells (i.e. fibroblasts, epithelial cells, endothelial cells, astrocytes, preadipocytes, and leukocytes as well as in postmitotic cells) participates, together the phenomenon of inflammageing, in the low chronic inflammation, improving both entropic ageing process and onset risk for age-related degenerative diseases, as result of harmful responses (i.e. chronic inflammatory responses, increased apoptotic resistance, decline in autophagic cleansing and tissue atrophy) (see Figure 2) [2,36,41]. Thus, chronic inflammation predisposes individuals to various age-related diseases. For example, the pro-inflammatory SASP of senescent endothelial cells has been proposed to contribute to CVD by initiating and fueling the development of atherosclerotic lesions. In addition, the expression of a SASP by astrocytes, which has been documented both in cells that were made senescent in culture as well as cells that were isolated from aged brain tissue, has been suggested to initiate or contribute to neuroinflammation, responsible of many neurodegenerative diseases, such as AD, causing or exacerbating age-related decline in both cognitive and motor function [42].

In the light of this evidence, the research has focused the attention on identifying pro-ageing factors, "*NF-κB activators*", as possible ageing biomarkers. Here, we describe some of them as promising inflammatory biomarkers of ageing and age-related diseases.

Oxidative stress, mitochondrial dysfunction, oxidative stress and activation of inflammasomes

Among the ageing modifications, mitochondrial alterations happen. They include an increased content of oxidation products and a diminished functional activity, conditions described and called as *mitochondrial dysfunction* [38,39]. An enhance of mitochondrial content in oxidation products, accompanies the entropic ageing process, and protein carbonyls, thiobarbituric acid reactive substances, ROOH and 8-hydroxy-2′-deoxyguanosine are the major markers. Recent experimental data on animal models, such as rats and mice, demonstrate the increase of these molecules with ageing in different tissues and organs [43]. In addition, current evidence underlines the increase of levels of several oxidative products in human biofluids, such as urine, serum, plasma, and blood, from old individuals than young subjects [44].

Mitochondrial dysfunction and oxidative stress are not associated only with ageing process, but also with the pathogenesis of several age-related diseases, as reported by recent experimental literature data [45-48]. Their detrimental effects are commonly attributed to disturbances in energy metabolism and increased Reactive oxygen species (ROS) production, and the crucial role of mitochondria in apoptotic cell death. In addition, mitochondria dysfunction and oxidative stress seem to provoke and potentiate inflammatory responses, even if the mechanisms remain elusive [38,39]. However, recent evidence sustains a crucial role of mitochondria in the regulation of innate immunity/inflammatory responses through different ways [49,50]. Among these, one is mediated by ROS which can induce the assembly of multi-protein inflammatory complexes

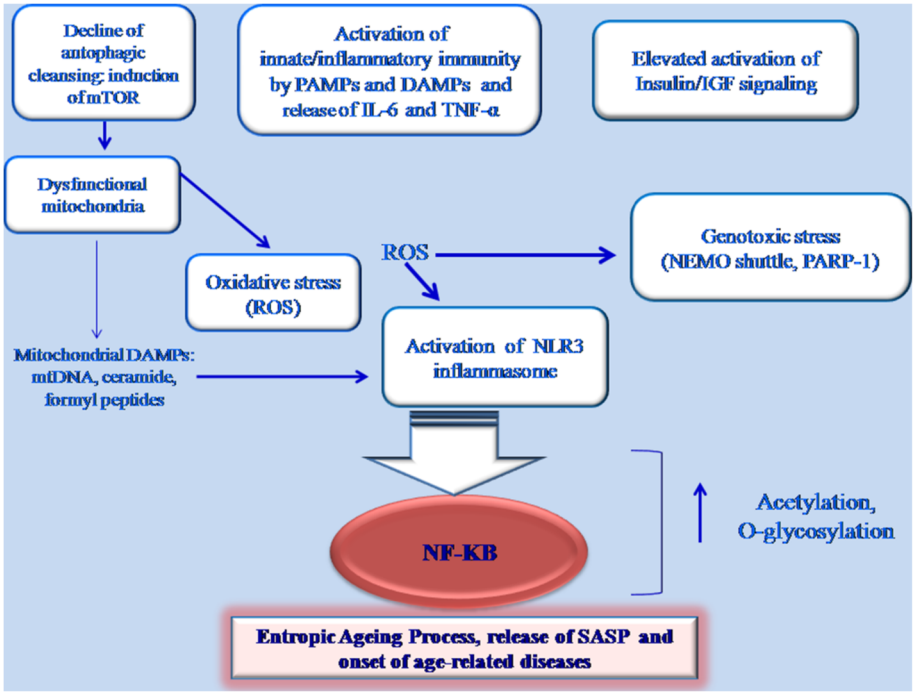

Figure 2 NF-kB system is at the hub of aging inflammatory network Its activation is induced by different factors, such as mitochondria dysfunction, oxidative stress, activation of Inflammasomes, decline of autophagic cleansing. Other NF-kB activators are activation of innate/inflammatory responses by PAMPs and DAMPs, elevated induction of insulin/IGF1 pathway, acetylation and O-glycosylation of components of NF-kB pathway, and DNA damage. NF-kB system induces entropic ageing process and release of SASP.

called inflammasomes [49,50]. In particular, they activate Nod-like receptor protein 3 (NLRP3), a member of these complexes and a major sensor of cellular stress signals, such as ROS. Subsequently, NLRP3 triggers the caspase-1 mediated maturation of precursors of IL-1β and IL-18 cytokines [51,52]. Thus, an endogenous stress-related inflammation is activated, defined by Medzhitov as "*para-inflammation*" [53]. The exact mechanism involved in the ROS-induced NLRP3 activation is still unclear. It has been recently demonstrated by Zhou and colleagues that ROS could activate NLRP3 inflammasomes via the redox regulation of thioredoxin/thioredoxin-interacting protein balance [54]. In addition, the ROS can directly activate the inflammasomal pathways through the oxidation of thiol groups in leucine-rich repeat domain of NLRP3. Furthermore, under loss of mitochondrial integrity, mitochondria secrete DAMPs, such as ROS, ceramide, mitochondrial DNA and formyl peptides, which can also provoke activation of NLRP3 inflammasomes local and para-inflammation responses [55,56].

Decline of autophagic function and induction of mTOR

As described above, mitochondria with disrupted integrity and a deficiency in cellular housekeeping can activate through different ways NLRP3, and also NLRP1 (another member of inflammasomes) in some tissues like brain, and stimulate inflammation [41,49,51]. In this context, the efficient function of autophagic uptake and lysosomal degradation of dysfunctional mitochondria should be a crucial element in maintaining tissue homeostasis [41]. Autophagy is, indeed, an ancient housekeeping mechanism, which regulates cellular homeostasis by facilitating the removal of misfolded proteins and dysfunctional organelles, such as mitochondria [57,58]. However, authophagic capacity seems to be compromised in ageing and age-related diseases, as proposed in "*garbage can*" hypothesis of Brunk and Terman [55]. On the other hand, there is growing evidence on inflammasome activation in many pathological conditions. Thus, a deficiency in authophagic housekeeping could trigger an inflammatory component and aggravate their pathogenesis [41,49,50,56-58]. After ten years of experimental work, the "garbage can" hypothesis still seems to be valid, since different research approaches have demonstrated clearly the decline of autophagy with ageing and the increased mitochondrial dysfunction [41,49,50,56-58]. Accordingly, the ageing decline in autophagy creates problems in cellular housekeeping functions, which stimulate NF-κB signaling directly or via inflammasomes trigger SASP and provoke the onset of entropic ageing phenotype [41]. Inflammatory NF-κB signaling seems also to have the capacity to repress

autophagy and to induce this destructive interplay between autophagy and inflammasomes [41,49,50,56-58]. In particular, TNF-α can induce or repress autophagy in a NF-κB dependent manner. In presence of NF-κB signaling, TNF-α activates mammalian Target of Rapamycin (mTOR), a major autophagy inhibitor. On the contrary, in cells lacking of NF-κB activation, TNF-α stimulates the expression Beclin 1, an enhancer of autophagy [41,49,50,56-58].

TOR is a highly conserved serine/threonine kinase and a central controller of cell growth, metabolism and ageing. mTOR is activated in response to nutrients, growth factors and cellular energy. Deregulation of mTOR has been implicated in inflammation, ageing and several age-related diseases (i.e. cancer, metabolic syndrome, neurological diseases) [59]. It interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2), differentially activated by distinct extracellular and intracellular signals. mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors and is acutely sensitive to rapamycin. It promotes cell growth by inducing and inhibiting anabolic and catabolic processes, respectively, and also drives cell-cycle progression. mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton. mTORC2 is insensitive to acute rapamycin treatment but chronic exposure to the drug can disrupt its structure. The activation of mTORC1 by growth factors and nutrients inhibits autophagy and promotes protein synthesis. Over time, this may promote cellular stress (protein aggregation, organelle dysfunction, and oxidative stress), which might lead to damage accumulation and a reduction in cell function and thus promote the development of aging-related diseases. Also, mTORC1 activation induces stem cell exhaustion, which reduces tissue repair and promotes tissue dysfunction [59].

Activation of innate/inflammatory response by PAMPs and DAMPs

During ageing, clonotypic immunity declines. This condition is defined *immune-senescence* [30]. In contrast, innate immunity seems to be efficiently activated and to induce a chronic inflammatory phenotype, as mentioned above [30]. The activation of innate immunity is mediated through the linking of pattern recognition receptors (PRRs), multi-ligand and evolutionarily conserved receptors (i.e. TLRs, NLRs and RIG-I-like receptors), with invading pathogen structures, called pathogen-associated molecular patterns (PAMPs), and endogenous danger molecules, the DAMPs [26]. This determines the release of different inflammatory mediators (i.e. IL-6 and TNF-α) by NF-κB pathway [25]. Among PRRs, TLRs, and mainly TLR4 and TLR2, recognize not only PAMPs, but also a large number of different alarmin age type DAMPs, including high mobility group box 1 (HMGB1), S100, heat shock protein (HSP)-60 and −70, and defensins [26,34]. In addition, both TLR2 and −4 have a key role in the pathogenesis of several age-related diseases [34]. Accordingly, variants of genes codifying these molecules seem to modify the susceptibility of age-related diseases and survival to extreme age, as recently described in our study [27]. On the other hand, the +896A/G (Asp299Gly; rs4986790) and +1196C/T (Thr399Ile; rs4986791) TLR4 SNPs have been phenotypically associated with changes in the production of pro- and anti-inflammatory cytokines, and principally the Asp299Gly SNP seems to have a key role in AD, prostate cancer, atherosclerosis and, reciprocally, in longevity [27,34].

Furthermore, an intriguing and innovative hypothesis has been recently suggested based on the crucial role of microRNAs in the dysfunction of TLRs signaling and the acquisition of SASP with NF-κB activation. Thus, these conditions can be considered as two interconnected phenomena [60].

During ageing, proteins, DNA and lipids, long-live macro-molecules can be targets of different age alterations. i.e. the Maillard reaction, a well known non-enzymatic glycosylation mechanism, induced as result of enhance of oxidative stress and hyperglycemia [2]. This results in the formation of protein glycation products, called AGEs (advanced glyaction end products), considered pro-ageing factors and activating NF-κB pathway by their linking with characteristic PRR receptors, the RAGE receptors (receptor for advanced glycation end products). With advancing age, AGE content increases in tissues. AGE process also improves in diabetes, atherosclerosis, neurodegeneration and several inflammatory diseases. The major harmful AGE effect in ageing seems to be the maintenance of anti-apoptotic and pro-inflammatory phenotype. Of special note is the glycation of collagen and elastin which seem to have a key role in vascular pathologies [26].

Induction of NF-κB signaling pathway by pro-inflammatory cytokines (mediated/or not by lipid rafts)

Activation of innate immunity in ageing process (see above) determines the production and release of SASP, such as different inflammatory molecules. Among these, pro-inflammatory cytokines are mostly observed to be at elevated level in elderly people. These cytokines can also activate the NF-κB pathway and this way can propagate and aggravate the inflammatory changes. IL-6 and TNF-α are clearly up-regulated with ageing, even if their exact role in the ageing process has been difficult to establish because of their complex and cell-type functions [25].

Recent evidence reported that NF-κB pathway activation via pro-inflammatory cytokines, and particularly via

TNF-α, can be mediated by lipid rafts. Precisely, the binding of TNF-α to the TNF receptor (TNFR) results in receptor clustering within specialized domains at the cell surface, named as *lipid rafts*, which function as physical platforms for various molecules and are involved in a variety of biologic processes, such as molecular sorting, membrane trafficking, and signal transduction. For example, lipid rafts are important in the primary steps of T-cell antigen receptor signaling and B-cell antigen receptor signaling via triggering the phosphorylation of adaptor proteins [61]. The dynamic recruitment of ligand-bound receptors into lipid rafts has been suggested to be critical for the initiation of signaling transduction, including the NF-κB pathway. Legler and colleagues [62] reported that translocation of TNFR to lipid rafts is essential for TNF-α-mediated NF-κB activation, and disturbances in lipid raft organization switch the effect of TNF-α signaling from NF-κB activation to apoptosis, demonstrating that lipid rafts are crucial for the outcome of TNF-α-activated signaling pathways. In addition, lipid rafts have also been demonstrated to be required for NF-κB activation induced by IL-1, lipopolysaccharides, CD40L, or CD3/CD28, and the disruption of lipid rafts results in the inhibition of NF-κB activation mediated by these stimuli [63-65]. These studies indicate that lipid rafts play important roles in the activation of proximal NF-κB signaling.

Excessive stimulation of insulin/ insulin like growth factor (IGF) signaling

An excessive insulin/IGF signaling has been demonstrated to accompany ageing process [2]. Insulin/IGF signaling determines detrimental age effects via NF-κB pathway evoking activation of IκB kinase α/β complex. As consequence improving of inflammatory responses and resistance of apoptosis are induced. Given that impairing the signaling of insulin/IGF signaling pathway can activate the FOXO-dependent lifespan extension, this implies the role of NF-κB pathway in driving the ageing process via insulin/IGF axis [66].

Post-translation modifications of the members of NF-κB pathway

Members of NF-κB pathway are targets of several post-translation modifications. They influence both the activation of the pathway and transcriptional efficiency of NF-κB system [2,25]. Phosphorylation and ubiquitination are the major regulatory changes during activation. Acetylation, O-glycosylation and sumoylation can also control the transcriptional efficiency of NF-κB system during stress condition., i.e. inflammatory responses. In addition, increased protein acetylation can also activate cellular senescence. On the other hand, molecules involved in cellular survival, such as the Sirtuin molecules (see below), and particularly SIRT 1 and 6, can deacetylate a NF-κB component, the p65, and repress NF-κB signaling [2,25].

Glucose tolerance decline, cause of insulin resistance and hyperglycemic disorders, determines O-glycosylation. Chronic hyperglycemia mediates glucotoxicity through AGE formation or via the production of O-linked N-acetylglucosamine (o-GlcNac)-modified proteins. On the other hand, levels of O-glycosylated proteins increase during ageing. In particular, an increased O- glycosylation of Ikkβ protein, able to enhance NF-κB activity, has been observed during ageing. O-glycosylation can also target p65 NF-κB protein and potentiate the transcriptional efficiency NF-κB components. This action is regulated by p53 protein, which can inhibit glycolysis and subsequently suppress the activation of Ikkβ/ NF-κB signaling [2,25].

DNA damages

One of the major stochastic age mechanism is genomic instability [2,67]. DNA lesions appear during ageing in both nuclear and mitochondrial DNA, as result of free radicals and oxidative stress. Under genotoxic stress, the major pathways activated are p53, NF-κB and PARP-1 (poly-(ADP-ribose)-polymerase-1) [67]. In particular, activation of NF-κB signaling represents one of the principal cellular features evoked by DNA damage [68]. The DNA damage-dependent NF-κB activation cascade is defined NEMO shuttle, since an essential NF-κB modulator (NEMO; as mentioned above) under genotoxic stress forms an complex with PIDD (p53-induced protein with a death domain) and RIP1 (receptor interacting protein) kinase [69]. This complex accumulates in nuclei and a nuclear matrix ligase (PIASy) can sumoylate the NEMO protein. Sumoylation is a prerequisite to allow a Ataxia telangiectasia mutated (ATM) kinase to phopshorylate NEMO protein. Subsequently, NEMO is desumoylated and the NEMO/ATM complex is exported from nuclei in cytoplasm where it activates Ikk kinases, by triggering NF-κB signaling. This consents to prevent the p53-induced apoptosis, since Ikk kinases phosphorylate p53 and induce its degradation by proteasomes [67-69].

Another hallmark of DNA damage is the induction of PARP-1 pathway, an ubiquitously expressed member of PARP family of enzymes able to modify proteins by poly (ADP-ribosyl)lation. PARP-1 is a sensor of DNA damage and maintains the genome integrity by regulating DNA repair [70]. In addition, PARP-1 is considered a novel co-activator of NF-κB signaling, which can potentiate the NF-κB activation in genotoxic stress [25,71]. Furthermore, it is one of the proteins involved in the regulation of the length of nucleoprotein structures located at the ends of chromosomes, the telomeres [71]. Telomeres are subject to shortening at each cycle of cell division and are highly sensitive to damage induced by oxidative

stress. During ageing, both chronic inflammation and oxidative stress induce increased base oxidation. In contrast, to the majority of genomic DNA, there is evidence that telomeric DNA is deficient in the repair of single strand breaks. Thus, it creates a persistent damage of telomeres and a faster rate of telomere shortening, which induces cellular senescence and a faster rate of biological ageing. Since chronic oxidative stress plays a major role in the pathophysiology of several chronic inflammatory diseases, it has been hypothesized that telomere length is reducing at a faster rate during oxidative stress. On the other hand, telomere shortening has been assumed a biomarker of premature cell senescence in vascular and metabolic diseases [71,72]. Therefore, assessment of telomere length as well as the evaluation of both function and integrity of PARP-1 might be useful biomarkers of both biological ageing and disease onset and progression [71,72].

Potential strategies against ageing and age-related diseases: drug and nutrition interventions and life-style modifications, and their effects on targets of inflammatory network

An excessive activation of NF-κB signaling pathway characterizes the entropic ageing process, responsible of inflamm-ageing and SASP phenotype, and the consequent onset of several age-related diseases [31-34,36]. This is plausible since nearly all insults enhancing the ageing process are well-known activators of NF-κB signaling system, as illustrated in Figure 2. The NF-κB signaling pathway also represents the lynchpin of host defense receiving the input signaling from the PRR receptors and subsequently organizing the transcriptional output response against the acute danger [25,26]. In both two cases, the sustained activation of NF-κB signaling pathway can trigger and enhance the entropic ageing process in many different ways, as above described [1,2]. Thus, the NF-κB system is at the hub of ageing process. This concept leads us in considering molecules and mechanisms linked to NF-κB signaling system as potential ageing biomarkers, as described. In addition, we also suggest them as targets for the development of new therapeutic strategies against ageing and age-related diseases.

On the basis of data reported herein, we proposed some suggestions on possible therapeutic drug and nutrition interventions and life-style modifications, and their effects on targets of inflammatory network (see Table 2).

Table 2 Potential therapeutic interventions and effects on targets of inflammatory network

Therapies	Target effects
Monoclonal antibodies against these cytokines and their receptors	Reduction of levels of IL-6, TNF-α
Non-steroidal anti-inflammatory drug	
Agonists of cytokine receptors or PRR receptors for people who do not respond to (or comply with) NSAID therapy	
Antibody-mediated stimulation of the decoy TLR receptors, such as TAM receptors, or of the intracellular TLR regulators for people with pro-inflammatory alleles in TLR4 and TLR2 genes	
Statin therapy	
Physical activity	
Administration of prebiotics and probiotics	
Caloric restriction	Decrease of oxidative stress
Polyphenols	
Use of drugs having mimic CR action	
Caloric restriction	Mitochondria biogenesis as preventive action against mitochondrial dysfunction
Use of drugs having mimic CR action	
Polyphenols	
Caloric restriction	Reduction of the activation of NF-KB pathway
Terpenoids	
Resveratrol	
Use of specific miRNAs	
Administration of prebiotics and probiotics	
Use of drugs having mimic CR action Curcumin	
Caloric restriction: inhibition of mTOR pathway	Preventive action on the possible reduced activity of autophagic cleansing
Rapamycin: inhibition of mTOR pathway	
Curcumin: influences the mTOR pathway	
Caloric restriction	Reduction of the excessive activation of Insulin/IGF1 pathway
Metformin with CR mimic response	

Anti-inflammatory drug interventions

➢***Use of monoclonal antibodies and/or non-steroidal anti-inflammatory drugs*** The presence of "high-risk" levels of IL-6 and TNF-α in elderly people suggests the possibility to develop preventive measures using specific inhibitors, such as monoclonal antibodies against these cytokines and their receptors. Reduction of inflammatory mediators may be also induced through non-steroidal anti-inflammatory drug (NSAID) therapy. For

people who do not respond to (or comply with) NSAID therapy, other more sophisticated preventive approaches may be possible, including the use of agonists of cytokine receptors or PRR receptors, i.e. TLR4 and −2, particularly in subjects carriers of high inflammatory responder alleles [27,34]. On the other hand, the activation of PRR receptors, such as TLR4 and −2, evocated by PAMPs or DAMPs particularly upon ageing, induce the release via NF-κB pathway of a large number of components of SASP, such as pro-inflammatory IL-6 and TNF-α cytokines [26,34]. In addition, the magnitude of cytokine production, and in general that of all pro-inflammatory mediators, has been shown to vary individually and is likely based on genetic heterogeneity. One or more functional SNPs in one or more innate immunty genes might be responsible. Accordingly, recent studies have suggested the role of +896A/G TLR4 SNP in cytokine production. In particular, high levels of pro-inflammatory cytokines were observed in carriers bearing the +896A/G TLR4 SNP [27,34].

Another possible therapeutic intervention in subjects with pro-inflammatory alleles of TLR4 and TLR2 genes might be antibody-mediated stimulation of the decoy TLR receptors, such as TAM receptors, or the intracellular TLR regulators (i.e. Supressor of cytokine signaling-SOCS molecules), involved in the inhibition of the inflammatory response, by mediating TLR degradation, or the activation of competitive or dephosphorylation functions [73]. The sequential induction of these pathways, and their integration with upstream TLR and cytokine signaling networks, may limit the inflammatory response and maintain innate immune system homeostasis. A better understanding of the regulatory mechanisms of this cascade may have important implications for therapeutic intervention in human immune disorders and reduce the risk development for several age-related diseases [27,34].

➢***Statin therapy*** Statin therapy has been demonstrated to have benefical effects in reducing primary and secondary CVD risk through the lipid-lowering, but also in inducing anti-ageing actions, such as inflammatory molecule lowering, especially IL-6 and CRP. On the other hand, results from Justification Trial Evaluating Rosuvastatin (JUPITER) confirmed that statin treatment in apparently healthy subjects with elevated CRP and non-elevated Low density lipoprotein cholesterol resulted in significant reduction in both these markers and CVD [74].

Nutrition interventions and life-style modifications

➢***Caloric restriction*** Another possible anti-ageing strategy, able to reduce the biological effects of NF-κB signaling pathway in ageing, is the notable caloric restriction (CR) [75]. Restricting the intake of calories has been practiced as a method for increasing both the length and quality of life for over 500 years. Experimental work confirming the success of this approach in animals has accumulated over the last 100 years. CR may extend life by up to 50% in rodents, with progressively less impact the later in life it is started. This effect is matched by profound impacts on age-related diseases, including reduced risk of cancer, neurodegenerative disorders, autoimmune disease, CVD and T2DM [75]. The disposable soma theory of ageing suggests that CR evolved as a somatic protection response to enable animals to survive periods of food shortage [4]. The shutdown of reproductive function during CR is consistent with this suggestion, but other features of the phenomenon are less consistent with this theory. Some researchers have, indeed, proposed that in rodents it may be mostly an artifact of domestication. CR induces profound effects on animals at all levels from the transcriptome to whole animal physiology and behavior. Animals under CR lose weight which is disproportionately contributed to by white adipose tissue. Generally animals on CR change their activity patterns. Thus, they are more active prior to food delivery each day, but total activity may be unchanged or reduced [75]. Considerable debate has occurred over the effects of CR on resting metabolic rate (RMR). Total RMR declines, but as body mass and body composition also change it is unclear whether metabolism at the tissue level also declines, is unchanged or even increases. Body temperature universally decreases. Hunger is increased and does not seem to decline even with very long term restriction. Circulating adipokines are reduced reflecting the reduction in white adipose tissue mass under CR [75]. There is also a large reduction in circulating insulin and glucose levels. There are profound tissue level changes in metabolism with a generalized shift from carbohydrate to fat metabolism.

Four pathways have been implicated in mediating the CR effects. They are the insulin/IGF-1signaling pathway, the Sirtuin pathway, the adenosine monophosphate (AMP) activated protein kinase (AMPK) pathway and mTOR pathway [75]. These different pathways may interact and all play important roles mediating different aspects of CR response. Exactly how they generate the health benefits remains open for debate. However, one of the major impact of CR is the reduction of oxidative stress [76]. As described above, the major cellular source of ROS are the mitochondria. Isolated mitochondria from animals under CR show a reduced ROS production. In particular, CR results in an increase in the level and activation of adenine nucleotide translocase and uncoupling proteins able to reduce the mitochondrial

membrane potential. This results in a decline in superoxide radical (O2) production and a less damage to the lipids in the mitochondrial membrane reduced ulteriorly by increases in the membrane lipid saturation [76]. Increases in superoxide dismutase convert superoxide into hydrogen peroxide and increased levels of Se-dependent glutathione peroxidase and catalase convert this to water reducing the production of the toxic hydroxyl radical (OH). Lowered levels of OH diminish the oxidative damage to proteins and DNA, which is further ameliorated by enhanced levels of degradation and base excision repair respectively [76]. Furthermore, CR induces mitochondrial biogenesis, as evidenced by changes in mtDNA levels, and protein levels [77]. Such effects on mitochondrial biogenesis are consistent with the idea that there may be a tissue level increase in oxygen consumption under CR which is accommodated in the reduced overall energy budget by the reduced amount of metabolizing tissue.

In addition, CR increases the levels of a member of Sirtuin family (SIRT1 to SIRT 7), NAD + dependent deactylases involved in the regulation of the activity of many proteins, energy metabolism, cell survival and longevity [78,79]. In particular, CR increases the expression of SIRT1 in multiple tissues, even if this effect does not appear to be uniform in all tissues or across different studies [80]. It has been demonstrated that SIRT1 interacts with p65/RelA protein and specifically cleaves the acetyl group form, the lysine-310 of p65 protein, involved in enhancing the trans-activation efficiency of NF-κB system [81,82]. Thus, SIRT1 is a potent inhibitor of NF-κB system.

An enhanced autophagy is also induced by CR via the inhibition of mTOR or the activation of AMPK pathway. This last is an evolutionary conserved sensor for disturbances in cellular energy balance and a major inductor of autophagy. Thus, CR acts directly or indirectly as inhibitor of NF-κB system [75,81,82].

Based on these observations, it is possible to assume that CR has beneficial effects, i.e. the extension of the average and maximum life span and delaying the onset of age-associated changes. However, this has been proven only in animal models, such as yeast, worms, flies and some mammals (rats and mice), and some criticisms (as above suggested) lead to consider it as an artifact of domestication, particularly in rodents [75,83-85]. In higher mammals, CR delays many diseases associated with aging including cancer, diabetes, atherosclerosis, CVD and neurodegenerative diseases [86,87]. The incidence of these diseases increases with age and they contribute significantly to mortality. Therefore, CR could increase life span by increasing the body's general state of health and providing a nonspecific, resistance to chronic diseases and metabolic derangements [86,87]. However, the ultimate question, how does CR effect the human body, was studied in a limited number of experiments [88]. The study of CR effects on human longevity faces ethical and logistical challenges since the average life span is close to 80 years for the population in developed countries. Therefore, human studies are focused on measuring the CR-related changes that could slow the aging process and the progression of chronic diseases thus increasing life span. The most convincing evidence that CR could have a positive effect in humans was provided by experiments by Fontana and coworkers, by the Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake (CALERIE Phase 1), and by data obtained on the members of the Caloric Restriction Society [89-93].

Fontana and coworkers [89] assessed the effect of a 6-year long CR diet on risk factors for atherosclerosis in adult male and female adults (age range 35–82 years) and compared them to age-matched healthy individuals on typical American diets (control group). The total serum cholesterol level and low-density lipoprotein (LDL) cholesterol levels, the ratio of total cholesterol to high-density lipoprotein cholesterol (HDL), triglycerides, fasting glucose, fasting insulin, CRP, platelet-derived growth factor AB, and systolic and diastolic blood pressures were all markedly lower in the CR group. The HDL cholesterol was higher after CR. Medical records of individuals in the CR group indicated that, before they began CR, they had serum lipid-lipoprotein and blood pressure levels in the expected range for individuals on typical American diets, and similar to those of the comparison group. Thus, this study concluded that long-term CR can reduce the risk factors for atherosclerosis.

The effect of fat loss induced by either (a) a long-term 20% CR or (b) a 20% increased energy expenditure (IEE) by exercise on coronary heart disease (CHD) risk factors was detected in a one-year randomized, controlled trial on 48 non-obese male and female subjects. The CR or exercise induced reductions in body fat were quantitatively similar and were accompanied by similar reductions in most of the major CHD risk factors, including plasma LDL-cholesterol, total cholesterol/HDL ratio, and CRP concentrations. Thus, these data evidenced that long-term CR or IEE of the same magnitude lead to substantial and similar improvements in the major risk factors for CHD in normal-weight and overweight middle-aged adults [90].

The effects of a 1-year, 20% CR regime or 20% IEE by exercise, on the oxidative damage of DNA and RNA, was evaluated by white blood cell and urine analyses in normal-to-overweight adults. Both interventions significantly reduced oxidative damage to both DNA and RNA in white blood cells compared to baseline. However, urinary levels of DNA and RNA oxidation products did

not differ from baseline values following either 1-year intervention program. The conclusion of the study was that either CR or IEE by exercise reduce systemic oxidative stress which is reflected in a decreased DNA or RNA oxidative damage [91].

CALERIE, a research program initiated by the National Institute on aging and involving three research centers, performed in the Phase 1 three pilot studies to determine whether long-term (6–12 months) effects of 20–25% CR in free-living, non-obese humans could be investigated and to evaluate the adaptive responses to CR. This randomized, controlled, clinical trial concluded that CR subjects had a lower body weight, a decreased whole body and visceral fat, a reduced activity energy expenditure, improved fasting insulin levels, improvements in cardiovascular disease markers (LDL, total cholesterol to HDL ratio, and CRP), and no change in bone density compared to controls [88]. In the ongoing CALERIE Phase 2, the researchers are testing whether 2 years sustained 25% CR of *ad libitum* energy intake results in beneficial effects, similar to those observed in animal studies [92].

Members of the Caloric Restriction Society (CRS) restrict food intake with the expectation that this would delay the disease processes responsible for secondary aging and to slow the primary aging process. Compared to age-matched individuals eating typical American diets, CRS members (average age 50 ± 10 yr) had a lower body mass index, a reduced body fat, significantly lower values for total serum cholesterol, LDL cholesterol, total cholesterol/LDL, and higher HDL cholesterol. Also fasting plasma insulin and glucose values were significantly lower than in the age-matched control group. Left ventricular diastolic function in CRS members was similar to that of about 16 years younger individuals. Chronic inflammation was reduced by CR and this was reflected in significantly lower levels of plasma CRP and TNF-α [88].

Aging is associated with a progressive reduction in heart-rate-variability (HRV)—a measure of declining autonomic function—and also a worse health outcome. The effect of a 30% CR on heart autonomic function was assessed by 24-hour monitoring of HRV in adults on self-imposed CR for 3 to 15 years and compared with an age-matched control eating a Western diet. The CR group had a significantly lower heart rate and significantly higher values for HRV. Also, HRV in the CR individuals was comparable to published norms for healthy individuals 20 years younger. The authors suggest that CR reset the balance between the sympathetic/parasympathetic modulation of heart frequency in favor of the parasympathetic drive thus increasing the circadian variability of heart rate [93].

Thus, in humans CR could delay many diseases associated with aging including cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative diseases. As an alternative to CR, several CR mimetics have been tested on animals and humans, as described below.

➢***CR mimetic drugs: biguanides, stilbenes and drugs*** Considerable effort has been directed in recent years to find drugs that mimic the CR response. Promising candidates are those that intersect with the critical signaling pathways identified above and include biguanides such as metformin, capable to target insulin signaling pathway, stilbenes (e.g. resveratrol) affecting sirtuin activity and drugs such as rapamycin that interact with mTOR signaling. Whether it will ever be possible to find drugs that capture the health benefits of CR without the negative side-effects remains unclear. Moreover, even if such drugs are developed how the current licensing system for drug use in western societies would cope with them may be a further obstacle to their use [75,88,94-96].

➢***Polyphenols and resveratrol (a stilbene phytochemical)*** As mentioned above, several plant derived, folk medical compounds and extracts have been claimed to have anti-ageing effects [75,88,97-101]. However, only a small number of traditional remedies has been subjected to a clinical trial. Recently, many promising compounds have been identified and scrutinized. Among these, there are polyphenols (i.e. flavonoids and terpenoids), the major ingredients of fruits, vegetables and different spices [75,88,97-101]. Many of polyphenols are inhibitors of NF-κB signaling system, since they are potent antioxidants, and as consequence they can inhibit ROS production and activation of NF-κB signaling system [97-101]. Some of them (i.e. terpenoids) can also directly inhibit Ikk/ NF-κB signaling [97-101]. Accordingly, it has been found that low-doses of terpenoids can trigger cellular stress response and subsequently induce adaptive stress resistance, condition defined hormesis [97-101]. Stress resistance involves several molecular adaptations via the activation of AMPK pathway and the subsequent increase in the expression of survival genes, such as Sirtuins, FOXOs and p53 [97-101]. Of special note is the effect of resveratrol, a stilbene phytochemical. It induces activation of SIRT1 via AMPK pathway, and indirectly inhibition of NF-κB signaling system via the activation of survival genes [97-101].

➢***Curcumin*** It has been postulated that a natural agent, curcumin, could influence cellular senescence [102]. Curcumin has attracted the attention of researchers and clinicians as an anti-inflammatory and anti-oxidant agent with a potential use in the therapy of many diseases with an inflammation constituent, e.g. cancer, CVD, AD, rheumatoid arthritis and metabolic syndrome. A plethora of studies using animal and cell line models have been undertaken to elucidate the molecular

mechanisms and biological effects of curcumin and some clinical trials are underway. Sikora and colleagues [103] proposed that curcumin might act as an anti-aging agent not only by inhibition of NF-κB, but also by indirect influence on cellular senescence via mTOR. However, they also showed that conversely curcumin can induce senescence in colon cancer cells [104]. Moreover recent studies by Quitschke [105] also revealed the prosenescence activity of curcumin. Nonetheless, curcumin has many molecular targets and evokes a biphasic hormetic dose–response [106]. Thus, one cannot exclude that much lower concentration of curcumin than that used in these studies will inhibit/postpone cellular senescence or, at least, through NF-kB inhibition will reduce SASP. Interestingly, curcumin was shown to prolong life of model organisms such as Caenorhabditis elegans and Drosophila melanogaster, but did not influence (similarly to resveratrol) the life span of mice [107].

➢***Physical activity*** Promising evidence suggests a role of physical activity in reducing the levels of inflammatory markers. Several speculations have been advanced [108-111]. However, the mechanisms underlying its anti-inflammatory effects seem complex and not fully elucidated. It has been recently considered that the decreased production of proinflammatory cytokines may originate from a reduction of adiposity, or the release of muscle derived IL-6 [108-111]. This last seems to induce several metabolic adaptations, i.e. hepatic glycogenolysis and lipolysis, and the release of cytokine inhibitors (i.e. IL-1ra, sTNFR and IL-10) and cytokine with potent anabolic effect, as IL-15 [108-111].

➢***Probiotics and/or prebiotics*** Administration of probiotics and/or prebiotics to elderly seems to induce changes in several inflammatory parameters (i.e. pro-inflammatory cytokine lowering, CRP reduction), demonstrating that manipulation of gut microbiota may result in modification of functionality of an aged immune system. On the other hand, intestinal microbiota seems to play a fundamental role in maintaining human health. Its supposed importance in human physiology has recently led to label human subjects as "metaorganisms" because of their close symbiotic relation with indigenous gut microbiota. The "metaorganisms" hypothesis evidences the use of dietary supplementation with probiotics and prebiotics, as therapeutic strategy to preserve human health particularly during that life period not foreseen by evolution- "ageing"-, that inexorably alters gut microbiota composition, stability and functionality [112,113].

Conclusions

From all observations described above, chronic inflammation has been proposed as the major biological mechanism underpinning the entropic ageing process and age-related diseases [31-34,36]. Inflammation is also the key response of host defense against pathogens and tissue injury [25,26]. In addition, it is current opinion that during evolution the host defense and ageing process have become linked together [2]. Thus, the large array of defense factors and mechanisms linked to the NF-κB system seem to be involved in the entropic ageing process [2,25]. This concept leads us in proposing inductors of NF-κB signaling system as potential ageing biomarkers and promising targets for the development of new therapeutic strategies against ageing and age-related diseases. In this report, we describe some inflammatory mechanisms linked to NF-κB signaling system as potential ageing biomarkers. In addition, some suggestions on their role as promising targets for the development of new therapeutic strategies have been discussed. Our interest has been, particularly, focused on possible interventions on molecular survival and resistance stress pathways, capable to reduce or inhibit NF-κB signaling pathway. However, it is not impossible to predict, whether these possible interventions (appropriate and specific drug therapies, lifestyle modifications, use of CR mimetics and other preventive therapeutic strategies) can very reduce or retard the onset of ageing biological phenotype and the onset risk of age-related diseases. Different motivations lead us to have prudence. Firstly, the major literature data on anti-ageing effects of therapeutic strategies have been obtained from studies on animals. Thus, potential therapy interventions on the basis of pathways identified in model organisms may be an illusion, because gains in longevity achieved in these organisms seem to decline with organismal complexity or depend on idiosyncratic physiology. Furthermore, lifespan in some organisms may be less plastic than in others. In addition, there are still enormous gaps in our knowledge about how metabolic pathways operate and interact. Serious side effects may constrain the effectiveness of pharmacological interventions.

The best treatment might be that which consents the repair of macromolecular damage. However, it is not clear that all toxic lesions associated with ageing process have been identified, or whether practical and appropriated strategies exist to eliminate them, as those mentioned above. Today, the researchers are becoming to speculate the concept based on reprogramming cellular senescence as way of organism rejuvenation or at least to alleviate age-related diseases considering cellular senescence as target model [114-116]. This hope derives by results of recent studies on progeroid mice demonstrating the possibility to reverse progeroid phenotype through genetic manipulation. This intervention of avoiding or reversing cellular senescence is based on induced pluripotent stem cell technology, which opened a

new avenue of autologous regenerative medicine and the possibility to activate telomerase and change the telomere length [117,118]. Accordingly, other studies are needed to confirm and extend these current data. For example, genomic, transcriptomic and epigenetic investigations may eventually lead to better understanding the molecular and cellular inflammatory mechanisms associated with biological ageing. In addition, for the development of anti-ageing therapies for human, it should be more appropriate identifying cellular and serum ageing biomarkers and potential targets using apposite study model, such as centenarian offspring, healthy elderly people with a family history of longevity, as recently suggested [119]. On the other hand, the research of biomarkers of ageing and age-related diseases in understanding the health trajectories of the oldest-old is unexplored territory. It is important that this lacuna is filled given the rapid growth in the number of very old people in many contemporary populations. The goal of this research might guarantee improving of life quality rather than searching the elixir of long life.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CRB wrote the drafts of this manuscript and revised the intellectual content; GC contributed to collection of literature data. CRB had the overall supervision of the review processing. All authors edited the paper and approved its final version.

Received: 23 December 2012 Accepted: 2 June 2013
Published: 20 June 2013

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doi:10.1186/1742-4933-10-24
Cite this article as: Balistreri *et al.*: NF-κB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies. *Immunity & Ageing* 2013 **10**:24.

5. Association of KLOTHO polymorphisms with healthy ageing: a systematic review and meta-analysis

ASSOCIATION OF KLOTHO POLYMORPHISMS WITH HEALTHY AGEING: A SYSTEMATIC REVIEW and META-ANALYSIS.

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Running Title: KLOTHO polymorphisms and ageing

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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.

Keywords: Ageing, Klotho, Longevity, Systematic review

INTRODUCTION

The ageing and longevity are multi-factorial events. Genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Approximately 25% of the overall variation in human lifespan 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 or multiple single nucleotide polymorphisms (SNPs) and ageing and longevity is greatly increasing even though a large number of these studies show inconsistent results (for an extensive analysis of "old" and recent data refers, to 2, 5). Consistent replication in different populations has been argued as strong evidence of a true association. However, the genetics of ageing 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, ageing and longevity phenotypes are strongly affected by life-style and environmental factors and by complex epistatic and pleiotropic effects in several genes (1, 6, 7).

GWAS consists in a scanning of whole genome analyzing 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 to analyze. In addition, population admixture may produce possible false positives due to different genetic backgrounds among ethnic groups. However, GWAS is an useful tool for the identification of complex trait associated alleles with frequency 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). With GWAS approach of long-living individuals, several loci have reached a genome wide significant level not always confirmed in different studies (3,8).

However, some interesting data have recently been obtained, such as those derived by meta-analysis (9). Meta-analysis 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 gave inconsistent results (4, 9).

The gene Klotho, aptly named after one of the Greek goddesses Fates, 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 fifteen years ago, as a gene which, if knocked out in mice, precipitates their accelerated ageing, including short lifespan, while its over-expression suppresses ageing and extends lifespan (10,11). On this basis, some human studies sought to demonstrate an association between the functional variant of 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 ageing 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 KLOTHO gene and human ageing 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, "ageing" and "longevity".

The abstracts found were read to identify studies examining the association between the above mentioned allele and ageing and longevity in healthy aged 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) case-control study; 2) there were at least two comparison groups of which one consisted of healthy aged or long-living individuals; 3) tested association between ageing and longevity and the variant.

Data extraction

Extraction of data was independently performed by DDB, GA and CV 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 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 fixed-effects and random-effects assumptions. The fixed effects model considers only within-study variability. The random effect 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 meta-analysis, 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 Klotho KL-VS variant and ageing have been 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) and thus it has been 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 2,913 aged people (from 199 to 723) and 2,206 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 Majumdar et al. study (15), we include in the meta-analysis only the healthy control population. Thus, people equal or under 40 are considered as the control population, whereas people over 40 are considered as aged (Table 1A). 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 the Table 1B. It should be noted that in the Invidia et al. study (14) a single SNP 115G>A, was used to tag KL-VS haplotype since all six SNPs occur in perfect linkage disequilibrium.

META-ANALYSIS

The effect for the KL-VS variant on ageing, suggested as favoring longevity, through a putative increase of secreted Khloto levels, has been estimated for each study. Five out of six studies (12A,B,C,14,15) show a little favorable effect on longevity, but only two of these reach the statistical significance (12A, 14). In contrast, the Novelli et al. study (13) show a little detrimental effect on longevity. The pooled summary OR for the genotypic comparison between the wild type (wt/wt) vs. the heterozygous (wt/KL-VS) variant is 1.14 (OR: 1.14, 95% C.I.: 1.00–1.30) with a marginal statistical significance (p=0.05) using the random-effects model (Figure 1A). In contrast, when we compare the Klotho wt homozygous subjects (wt/wt) vs. KL-VS-bearing genotypes

(wt/KL-VS + KL-VS/KL-VS), the summary OR is 1.10 (95% C.I.: 0.97–1.25; p=0.13), suggesting that KL-VS homozygous subjects not only do not show any advantage in ageing, but seem to have a disadvantage (Figure 1B), although this comparison does not reach the statistical significance. This observation seems to be confirmed by comparing KL-VS homozygous people (KL-VS/KL-VS) vs. Klotho wt homozygous + heterozygous subjects (KL-VS/wt + wt/wt) (OR: 0.73; 95% C.I.: 0.41–1.30; p=0.29), but, also in this case, with a statistically not significant result (Figure 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 Klotho KL-VS variant and ageing and longevity, representing a pooled total of 2,913 cases and 2,206 controls. The results indicate a significant association of the variant with healthy ageing 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, while 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 19-65 year (14). There are many differences also between the ageing populations, leading, in some cases, to an overlapping between cases and controls of different studies (Table 1A).

The populations included in the analysis are from different ethnicity. However, a subgroup analysis exploring the effect on 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 ageing and longevity compared to the Klotho wild type gene, the KL-VS variant in homozygosis show 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 (Figure 1C) which 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 (Figure 1C), with studies showing conflicting results, differently from the other comparisons (Figures 1A, 1B). 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 Invidia et al. study (14) it has been demonstrated that the KL-VS variant has been observed to be increased in 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 decreasing thereafter, and becoming no more evident at the extreme ages.

Concerning the function of Klotho leading to such effects on healthy ageing 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 lifespan of Klotho knockout mice is rescued by the Insulin/IGF-1 pathway inactivation (19). Thus, it is possible that secreted Klotho protein may modify glycans of the insulin/IGF-1 receptors that inhibits their activity and/or alters cell surface abundance (10,11).

The inhibition of this pathway has a critical role in the determination of longevity and several variants which 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 the phosphatidylinositol-3'kinase 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 anti-oxidant catalase and mitochondrial manganese-superoxide dismutase (11,20), hence the inhibition of this pathway

induces resistance to oxidative stress.

We can conclude that KL-VS variant, that influences the trafficking and catalytic activity of the secreted protein, favors healthy ageing and longevity inhibiting Insulin/IGF-1 signaling pathway. In fact, adequate suppression of this pathway is an evolutionarily conserved mechanism for anti-ageing and lifespan extension since this pathway negatively regulates transcription factors FOXO involved in upregulation of homeostatic genes (9-11,20).

CONFLICT OF INTERESTS

The authors have no conflict of interest.

ACKNOWLEDGMENTS

This meta-analysis has been 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.

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LEGEND TO FIGURE

Figure 1A. Meta-analysis of six case-control studies of the Klotho KL-VS polymorphism and ageing using the random-effects model. The odds ratio and 95% confidence interval (CI) for the effect of the wt/KL-VS vs. wt/wt genotypes on ageing 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.

Figure 1B. Meta-analysis of six case-control studies of the Klotho KL-VS polymorphism and ageing using the random-effects model. The odds ratio and 95% confidence interval (CI) for the effect of the KL-VS/KL-VS+ wt/KL-VS vs. wt/wt genotypes on ageing 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.

Figure 1C. Meta-analysis of six case-control studies of the Klotho KL-VS polymorphism and ageing using the random-effects model. The odds ratio and 95% confidence interval (CI) for the effect of the KL-VS/KL-VS vs. wt/KL-VS+ wt/wt genotypes on ageing 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.

TABLE 1. A. Clinical characteristics of the populations included in the meta-analysis

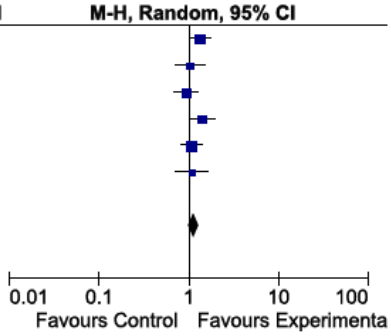
Study (year of publication)	Population	Cases		Controls	
		n	Age	n	Age
Arking et al. (2002)	Bohemian Czech	415	≥75	390	newborn
Arking et al. (2002)	Baltimore Caucasian	723	≥65	420	newborn
Arking et al. (2002)	Baltimore African-American	242	≥65	226	newborn
Novelli et al. (2008)	U.S. Caucasian	708	93-105	332	<35
Invidia et al. (2010)	Italian	626	66-109	463	19-65
Majumdar et al. (2010)	Indian	199	>40	375	<40

TABLE 1B. KLOTHO genotypes in case and control population

Study (year of publication)	Population	wt/wt	wt/KL-VS	KL-VS/KL-VS	n
Arking et al (2002)					
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		169	68	5	242
controls	Baltimore African-American	156	58	12	226
Novelli et al (2008)					
Cases	U.S. Caucasian	517	170	21	708
controls		241	85	6	332
Invidia et al (2010)					
cases	Italian	439	174	13	626
Controls		348	103	12	463
Majumdar et al (2010)					
cases	Indian	140	53	6	199
controls		270	99	6	375

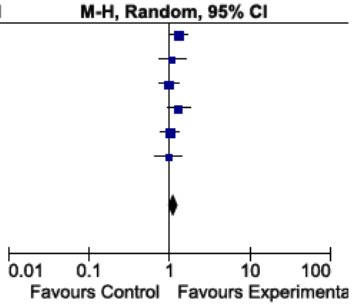
Study or Subgroup	WT/KL-VS Events	Total	WT/WT Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Invidia 2010	174	277	439	787	22.0%	1.34 [1.01, 1.77]
Majumdar 2010	53	152	140	410	11.4%	1.03 [0.70, 1.53]
Novelli 2008	170	255	517	758	19.1%	0.93 [0.69, 1.26]
Arking (A) 2002	103	176	308	615	15.2%	1.41 [1.00, 1.97]
Arking (B) 2002	185	285	530	839	22.1%	1.08 [0.81, 1.43]
Arking (C) 2002	68	126	169	325	10.2%	1.08 [0.72, 1.63]
Total (95% CI)		**1271**		**3734**	**100.0%**	**1.14 [1.00, 1.30]**
Total events	753		2103			

Heterogeneity: Tau² = 0.00; Chi² = 4.89, df = 5 (P = 0.43); I² = 0%
Test for overall effect: Z = 1.95 (P = 0.05)



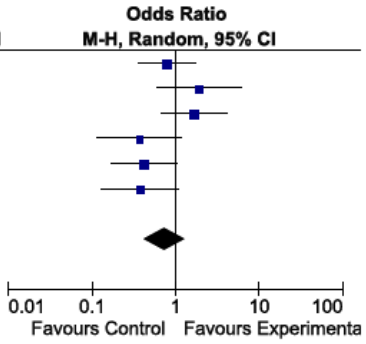
Study or Subgroup	WT/KL-VS + KL-VS/KL-VS Events	Total	WT/WT Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Invidia 2010	187	302	439	787	22.1%	1.29 [0.98, 1.69]
Majumdar 2010	59	164	140	410	11.3%	1.08 [0.74, 1.58]
Novelli 2008	191	282	517	758	19.0%	0.98 [0.73, 1.31]
Arking (A) 2002	107	190	308	615	15.2%	1.28 [0.93, 1.78]
Arking (B) 2002	193	304	530	839	21.9%	1.01 [0.77, 1.33]
Arking (C) 2002	73	143	169	325	10.5%	0.96 [0.65, 1.43]
Total (95% CI)		**1385**		**3734**	**100.0%**	**1.10 [0.97, 1.25]**
Total events	810		2103			

Heterogeneity: Tau² = 0.00; Chi² = 3.58, df = 5 (P = 0.61); I² = 0%
Test for overall effect: Z = 1.51 (P = 0.13)



Study or Subgroup	KL-VS/KL-VS Events	Total	KL-VS/WT + WT/WT Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Invidia 2010	13	25	613	1064	20.3%	0.80 [0.36, 1.76]
Majumdar 2010	6	12	193	562	14.3%	1.91 [0.61, 6.01]
Novelli 2008	21	27	687	1013	18.0%	1.66 [0.66, 4.15]
Arking (A) 2002	4	14	411	791	14.0%	0.37 [0.12, 1.19]
Arking (B) 2002	8	19	715	1124	17.9%	0.42 [0.17, 1.04]
Arking (C) 2002	5	17	237	451	15.6%	0.38 [0.13, 1.09]
Total (95% CI)		**114**		**5005**	**100.0%**	**0.73 [0.41, 1.30]**
Total events	57		2856			

Heterogeneity: Tau² = 0.25; Chi² = 10.09, df = 5 (P = 0.07); I² = 50%
Test for overall effect: Z = 1.06 (P = 0.29)



6. Association between genetic variations in insulin/insulin-like growth factor (IGF-1) signaling pathway and longevity: a systematic review and metaanalysis

Curr Vasc Pharmacol. 2013 Dec 18. [Epub ahead of print]

ASSOCIATION BETWEEN GENETIC VARIATIONS in the INSULIN/INSULIN-LIKE GROWTH FACTOR (IGF-1) SIGNALING PATHWAY and LONGEVITY: a SYSTEMATIC REVIEW and META-ANALYSIS.

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Running Title: IGF pathway-related gene polymorphisms and Longevity

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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.

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

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 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 evolutionary conserved correlation with life-span extension in model animals, such as yeast, nematodes, fruit flies and mice (Figure 1). Indeed, 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 obtained 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 IGF-1 circulating levels [3]. In human beings, ageing is associated with lower IGF-1 circulating levels [5], and in longevous people 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 (Figure 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 orthologue 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 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 (Figure 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 the 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, independently from 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 independently performed by GA, CV and DDB 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 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 fixed-effects and random-effects assumptions. The fixed effects model considers only within-study variability. The random

effect 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 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 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. The remaining study was conducted on Ashkenazi Jews in North America. All the analyzed studies had 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 was not reported, while in the Suh et al. study only females were studied [6]. The study sample size varied from 240 to 671 [6, 10]. 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, which was conducted on Japanese and the Pawlikowska et al. study, which was conducted on Ashkenazi. The Willcox et al. study was conducted only on male subjects; the Anselmi et al. and Soerensen et al. studies analyzed separately data from males and females. The Pawlikowska et al. study did not analyze separately data from males and females. The Flachsbart et al. study did not report males/females 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 males/females 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 were available for the inclusion in the meta-analysis of the association between the rs2229765 SNP (3174 G>A) and longevity [6, 8-10]. The effect for the A allele and for the A-bearing genotype of IGF-1R, suggested as favoring longevity, was estimated for each study. Regarding the allelic comparison (A vs. G), three out of four studies showed a favorable effect on longevity, while, the Bonafè et al. study showed a detrimental effect on

longevity [6, 8,-10] (Figure 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 not 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 statistically significant result using the random-effects model, suggesting that subjects with low IGF-1 level associated genotypes (AA or AG) have greater chance to attain 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%) (Figure 2A and 2B). The Bonafè et al. study is the influential one for the allelic comparison, since removing this the heterogeneity change from 82% to 0%, while the Suh et al. study is the influential one for the genotypic comparison, since removing this the heterogeneity change from 68% to 0%. The exclusion of the Suh et al. study from the genotypic analysis did 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 from the allelic comparison change 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 not 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 were available for the inclusion in the meta-analysis of the association between the rs2764264 and rs2802292 SNPs and longevity [13, 15, 16]. Data were suitable only for allelic comparison. For the rs2764264 we report a statistically not 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%) (Figure 3A), but a statistically significant results when only males were 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 (Figure 3B). For the rs2802292 SNP, we report statistically significant results for an association between the G allele and longevity, when we compared both the overall 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 reported data only for males [13, 16].

Data for the rs7762395, rs9400239 and rs479744 were reported in two studies, the Soerensen et al. and Flachsbart et al. studies [14, 15]. For the rs7762395, we do not report 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: 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 males LLI from the Soerensen et al. study were 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 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, nematode and fruit fly [3, 24]. Figure 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 increase 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 mouse have 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 a 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 a substantial but not conclusive evidence of an effect in some genes of this pathway on achievement of longevity. So, in the present paper we performed a systematic review and meta-analysis with the aim to reconcile the study inconsistencies.

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

No association was reported between the SNPs of IGF-1 and longevity in the available study 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].

Available data from four studies [6, 8-10], show, instead, a statistically significant association of longevity with the IGF-1R polymorphism rs2229765, suggesting that subjects with the A-bearing genotype responsible for a reduced signal transduction have higher chance of attaining longevity. The relevance of IGF-1R for longevity is further suggested by a meta-analysis performed on participants from 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 significant association with longevity was observed for the C allele when only males were

included in the analysis. The same was true for other SNPs, i.e. rs2802292, G allele (but for this allele 2 out of 3 studies reported 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 were significantly associated with longevity in centenarian population, as well as rs1935949 and rs4946935 SNPs. Also 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 an 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 might be pharmacologically modulated. However, it has to be clear that we have no understanding in which directions these SNPs could possibly act.

In any case, extensive studies of the whole pathway are needed, including the recent reported gene codifying calcium/calmodulin-dependent protein kinase IV (CAMKIV). In fact, 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 INTERESTS

The authors have no conflict of interest.

ACKNOWLEDGMENTS

This meta-analysis was entirely supported by the authors' respective institutions.

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LEGENDS TO FIGURES

Figure 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 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).

Figure 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.

Figure 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.

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 1.

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] Suh et al '08[c] Albani et al '09[d] Barbieri et al '12[e]	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
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]	G>A	1066/2020	OR (All) = 1.17 (95%CI 0.95, 1.43)	3/0	No significant association

	Soerensen et al '10[h]					
	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
		Soerensen et al '10[h]		1344/1102	OR (Males)* = 1.20 (95%CI 1.01, 1.43)	2/0	
	-Centenar.		T>C	531/1467	OR (All) = 1.32 (95%CI 1.06, 1.64)	2/0	Significant association in centenarians
		Flachsbart et al (GER) 09[i]		418/1102	OR (Males)* = 1.37 (95%CI 1.07, 1.76)	2/0	
		Soerensen et al '10[h]					
	rs479744	Flachsbart et al					
	-LLI	(GER) '09[i]	A>C	2120/1467	OR (All) = 1.16 (95%CI 0.99, 1.37)	2/0	No significant association
		Soerensen et al '10[h]		1344/1102	OR (Males)* = 1.22 (95%CI 1.00, 1.48)	2/0	
	-Centenar.	Flachsbart et al (GER) '09[i]	A>C	531/1467	OR (All) = 1.41 (95%CI 1.11, 1.79)	2/0	Significant association in centenarians
				418/1102	OR (Males)*= 1.41 (95%CI 1.07, 1.86)	2/0	
		Soerensen et al '10[h]					
	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

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

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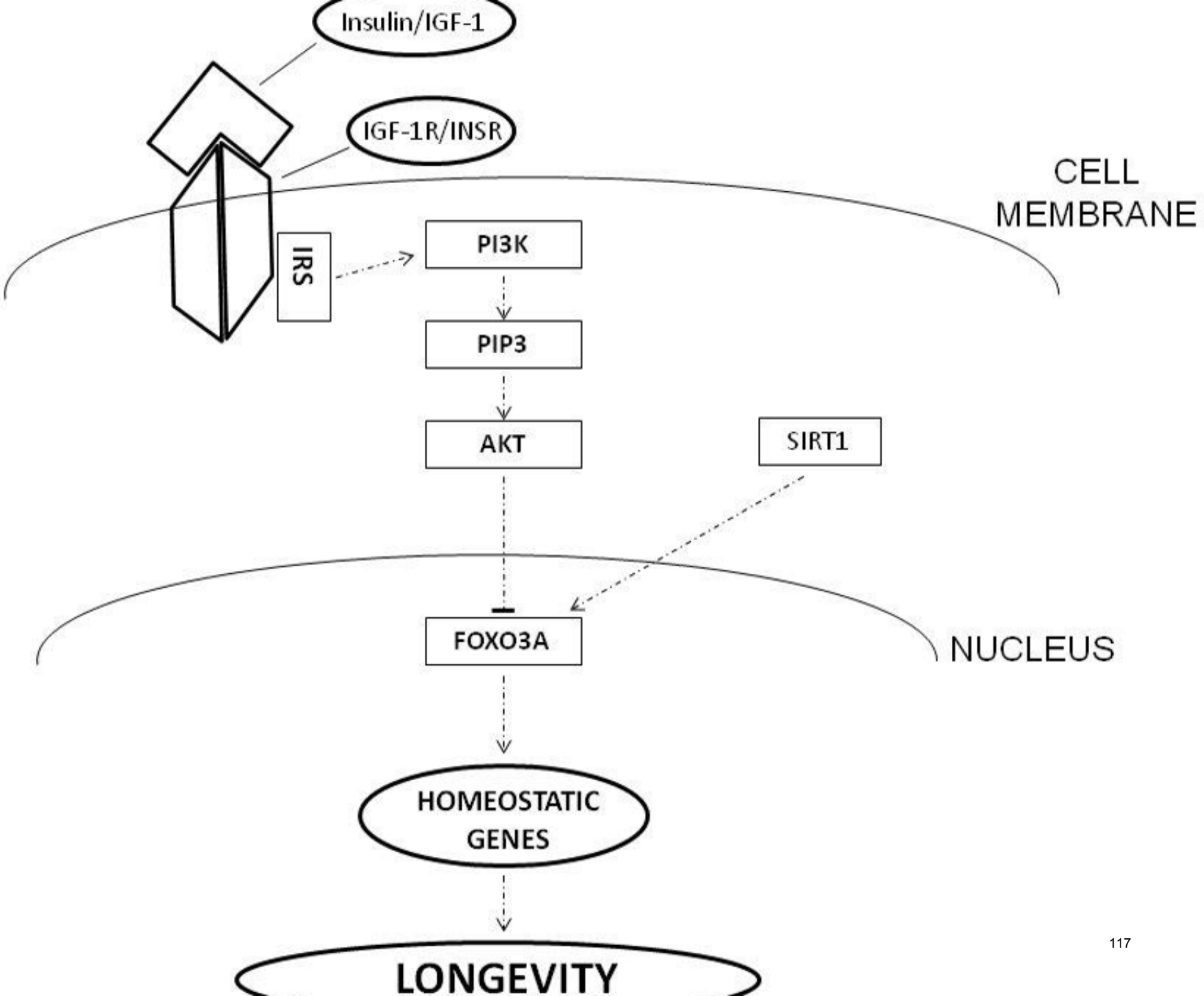
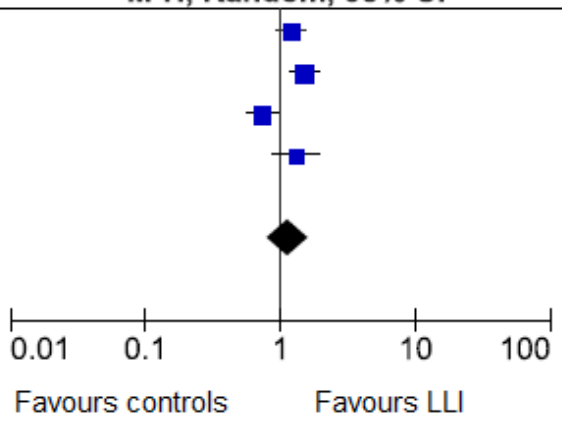

Insulin/IGF-1
IGF-1R/INSR
CELL MEMBRANE
IRS
PI3K
PIP3
AKT
SIRT1
FOXO3A
NUCLEUS
HOMEOSTATIC GENES
LONGEVITY

Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Albani 2009	194	444	225	576	26.2%	1.21 [0.94, 1.56]
Barbieri 2012	190	366	408	976	26.6%	1.50 [1.18, 1.91]
Bonafè 2003	159	324	397	696	25.8%	0.73 [0.56, 0.95]
Suh 2008	88	158	125	255	21.4%	1.31 [0.88, 1.95]
Total (95% CI)		**1292**		**2503**	**100.0%**	**1.14 [0.82, 1.59]**
Total events	631		1155			

Heterogeneity: Tau² = 0.09; Chi² = 16.91, df = 3 (P = 0.0007); I² = 82%

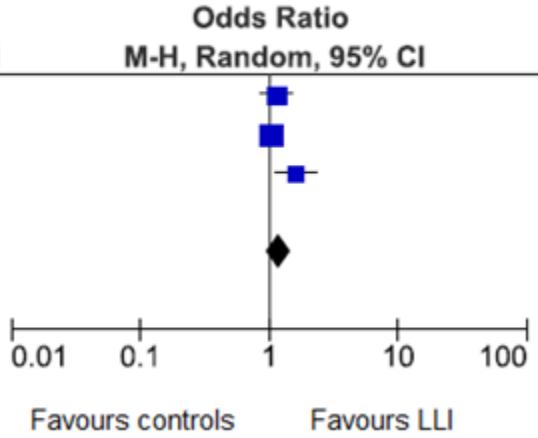
Test for overall effect: Z = 0.78 (P = 0.43)



Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Anselmi 2009	201	480	129	335	32.5%	1.15 [0.87, 1.53]
Soerensen 2010	320	1089	210	736	42.5%	1.04 [0.85, 1.28]
Willcox 2008	74	213	100	402	25.0%	1.61 [1.12, 2.31]
Total (95% CI)		**1782**		**1473**	**100.0%**	**1.20 [0.95, 1.51]**
Total events	595		439			

Heterogeneity: Tau² = 0.02; Chi² = 4.17, df = 2 (P = 0.12); I² = 52%

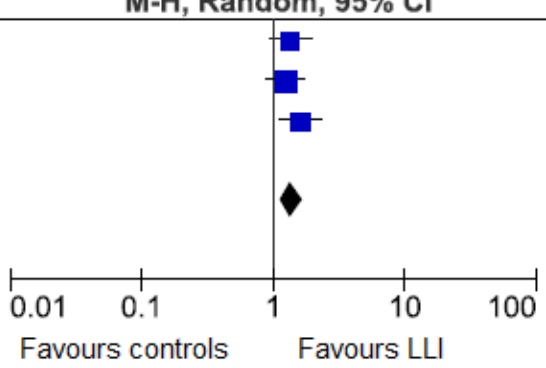
Test for overall effect: Z = 1.54 (P = 0.12)



Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Anselmi-M 2009	121	281	70	195	29.5%	1.35 [0.93, 1.97]
Soerensen-M 2010	101	313	103	371	38.7%	1.24 [0.89, 1.72]
Willcox 2008	74	213	100	402	31.9%	1.61 [1.12, 2.31]
Total (95% CI)		**807**		**968**	**100.0%**	**1.38 [1.13, 1.69]**
Total events	296		273			

Heterogeneity: Tau² = 0.00; Chi² = 1.11, df = 2 (P = 0.57); I² = 0%

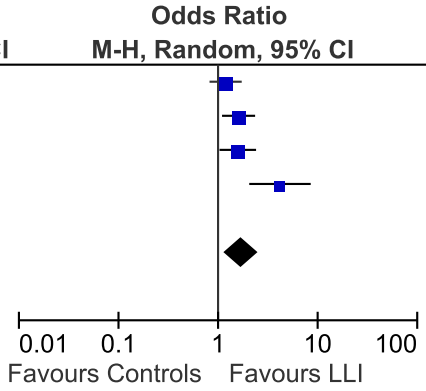
Test for overall effect: Z = 3.10 (P = 0.002)



Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Albani 2009	152	222	186	288	28.4%	1.19 [0.82, 1.73]
Barbieri 2012	137	183	317	488	28.0%	1.61 [1.10, 2.35]
Bonafè 2003	114	162	149	248	26.5%	1.58 [1.03, 2.41]
Suh 2008	68	79	96	161	17.1%	4.19 [2.06, 8.52]
Total (95% CI)		**646**		**1185**	**100.0%**	**1.73 [1.16, 2.58]**
Total events	471		748			

Heterogeneity: Tau² = 0.11; Chi² = 9.48, df = 3 (P = 0.02); I² = 68%

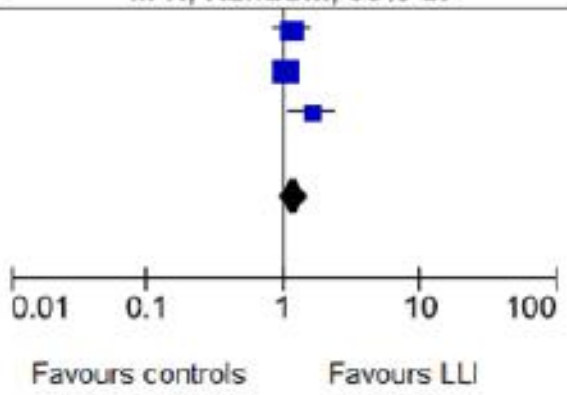
Test for overall effect: Z = 2.70 (P = 0.007)



Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
Anselmi 2009	201	480	129	335	32.5%	1.15 [0.87, 1.53]
Soerensen 2010	320	1089	210	736	42.5%	1.04 [0.85, 1.28]
Willcox 2008	74	213	100	402	25.0%	1.61 [1.12, 2.31]
Total (95% CI)		**1782**		**1473**	**100.0%**	**1.20 [0.95, 1.51]**
Total events	595		439			

Heterogeneity: $Tau^2 = 0.02$; $Chi^2 = 4.17$, df = 2 (P = 0.12); $I^2 = 52\%$

Test for overall effect: Z = 1.54 (P = 0.12)



Study or Subgroup	Long lived Events	Total	Controls Events	Total	Weight	Odds Ratio M-H, Random, 95% CI
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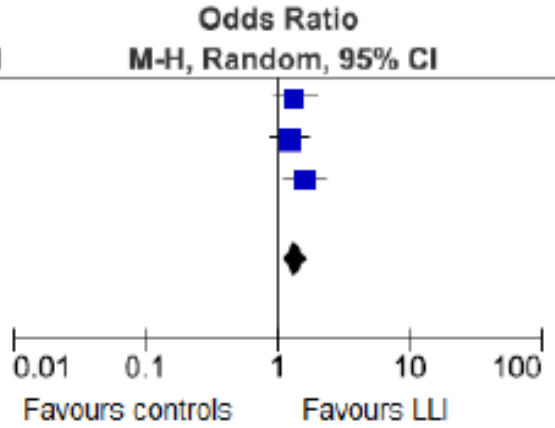
Willcox 2008	74	213	100	402	31.9%	1.61 [1.12, 2.31]
Total (95% CI)		**807**		**968**	**100.0%**	**1.38 [1.13, 1.69]**
Total events	296		273			

Heterogeneity: Tau² = 0.00; Chi² = 1.11, df = 2 (P = 0.57); I² = 0%

Test for overall effect: Z = 3.10 (P = 0.002)



7. SHIP2: a “NEW” insulin pathway target for ageing research

SHIP2: a "NEW" insulin pathway target for ageing research

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Running Title: SHIP2 polymorphisms and ageing

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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.

Keywords: Ageing, Alzheimer's disease, Insulin pathway, Longevity, SHIP2, Type 2 Diabetes

INTRODUCTION

Ageing 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. Insulin/Insulin Growth Factor(IGF)-1 pathway seems to be correlated to human life-span and its homologous 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 insulin signaling pathway, but, molecular mechanisms implicated are not so clear, although inflammatory process is involved. IR is one of the features of metabolic syndrome (MS), a pre-diabetic status [4, 5].

Interestingly, strong evidence suggests that systemic inflammation and central adiposity contribute to and perpetuate MS. 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 while each of the corresponding growth factors is normally made in central nervous system neurons, the expression levels are markedly reduced in AD [13].

Nowadays many evidences demonstrate 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 (Aβ 40–42 amino acids) and neurofibrillary tangles originating from

hyperphosphorylation of microtubular tau protein. The amyloid hypothesis is not the unique for the pathogenesis of AD. Indeed, different pathophysiological theories exist focusing the attention on inflammation, vascular changes and metabolic disorder. The most plausible hypothesis is that all 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 MS. Indeed, dementia and MS present some overlap both in predisposition factors, such as diet, smoking, socio economic status and life style and in altered signaling cascades, i.e. nutrient sensing pathway as the insulin one [18].

The Src homology domain-containing inositol 5-phosphatase(SHIP)2, has an important role in insulin pathway. It leads to the activation of AKT, acting on glycogen synthase kinase (GSK)3 (Figure 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 neuropathology of AD [21, 22]. Many studies underline the role of SHIP2 as 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], finding partly confirmed by another study [27]. Moreover, a study conducted in non-T2DM subjects with hypertension (one of the features of MS previous associated with the SNPs), found no association, identifying the T2DM as condition probably necessary for the association [28].

Starting from all 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, the rs9886 and the rs144989913.

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. On the whole, we collected 468 whole blood samples in EDTA Vacutainer.

Specifically, we enrolled 127 unrelated young (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 based on 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 NINCDS/ADRDA and DSM-III-R 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]. 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 enrolled as cases, as well. 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, that is an insertion/deletion (I/D) of 28 base pair, by classic PCR and the 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 χ^2 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 χ^2 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. For the rs9886 we did not find any association, 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 heterozygous genotype was increased in T2DM and AD patients as well as in old and centenarians 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 is observed. There are 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 highlight, with a 3x2 table, a growing significant increase (P=0.0016) of D with increasing age (young= 0,39; old=0,11; centenarians=0,15).

Gender analysis demonstrate that the significant difference in the rs144989913 genotypic and allele frequencies between T2DM and young, AD and young and old and young are present only in males rather than in both males and females (data not shown). Due to small number of centenarians, we couldn't study the gender effect in this population.

DISCUSSION

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

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 D allele increase from young to centenarians. Therefore, in a further step it should be analyzed the life expectancy in aged patients with D allele in comparison with 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 demonstrated the association of insulin/IGF1 pathway with ageing and longevity. The replication of specific results in model organisms led to conduct studies also in human [3,33].

It is tempting to speculate that rs144989913 alleles may differently influence gene expression because they consist in a variation of 28 base pair. They may differently modulate the insulin pathway involved in ageing and longevity, hence functional studies are mandatory to confirm this suggestion.

In conclusion, our results are only a small contribute in ageing research but represent the first study that coupled INPPL1/SHIP2 and ageing. INPPL1 might be a "new" interesting gene in ageing research and this study represent the first tile.

CONFLICT OF INTERESTS

The authors have no conflict of interest.

ACKNOWLEDGMENTS

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

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

Table 1. rs144999813 genetic distribution and allele frequency for cases represented by Alzheimer's 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.

*The significance of the different genotype distribution among groups was calculated by chi-square test (3x2 table).

**The significance of the different allele distribution among groups was calculated by chi-square test (2x2 table).

T2DM: Type 2 diabetes mellitus. AD: Alzheimer's disease. I: insertion. D: deletion.

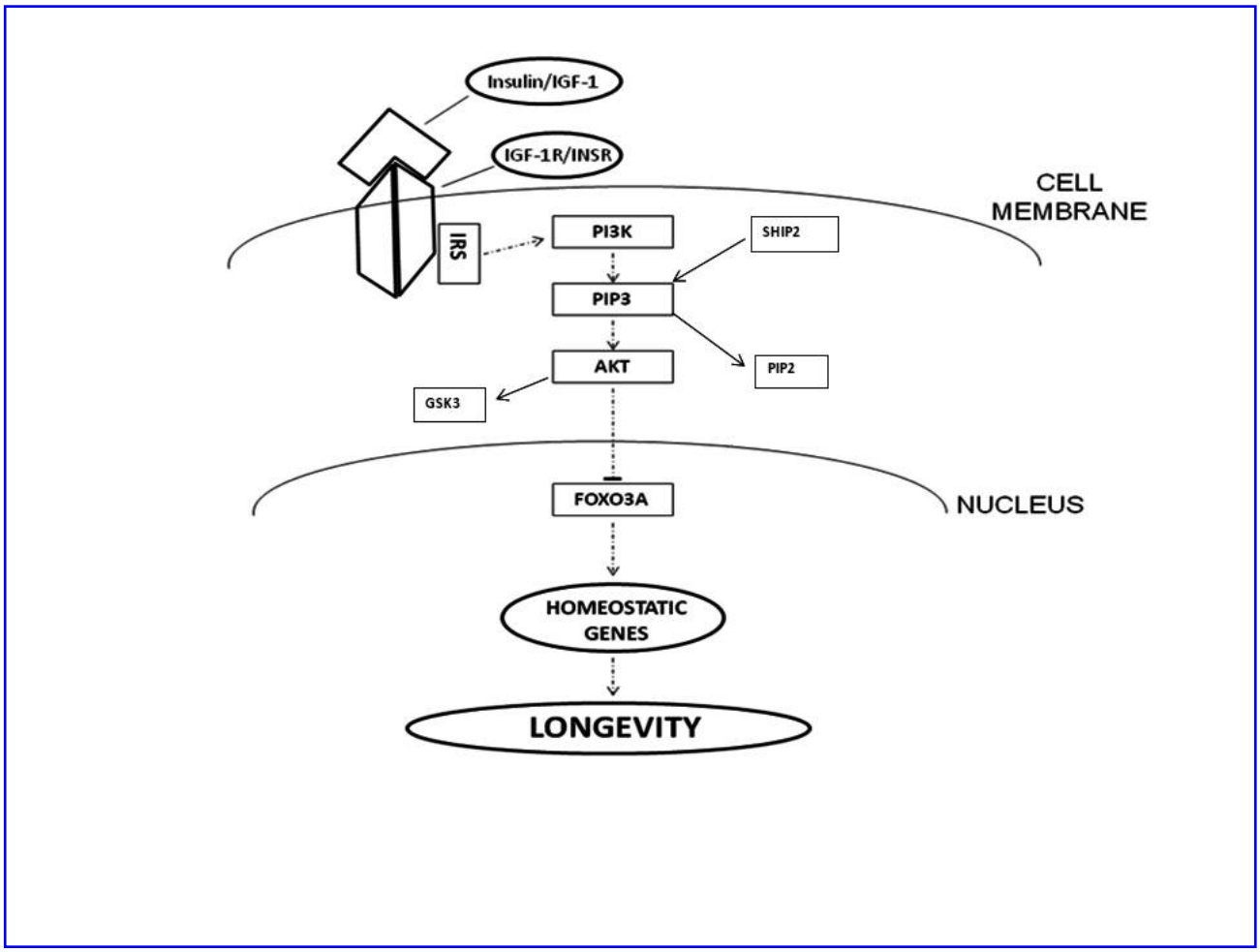

Figure 1. Insulin/IGF-1 pathway and SHIP2 action. This signaling pathway has a critical role in the determination of longevity. The bond of Insulin/IGF-1 to the specific receptor (IGF-1R/INSR) activates the phosphatidylinositol-3'kinase (PI3K) through the insulin related substrate (IRS). It leads to the activation of AKT, through phosphatidylinositol(3,4,5)-trisphosphate, that, in turn, inhibits Forkhead box O3A (FOXO3A), that 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/PKB that 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.

8. DISCUSSION AND CONCLUSION

The present thesis strengthen the suggestion of the central role played by insulin/IGF-1 pathway in human ageing and longevity.

Human population is very heterogeneous because of the different genetic background and different environmental stimuli thus it has not been yet possible to identify a clear panel of biomarkers of ageing and longevity.

Longevity is a complex trait influenced by familial component and other determinants.

Besides environmental factors (diet, physical activity, health habits, socio-cultural factors and life-style in general), genetic differences contribute at least for 25% in different human lifespan (*Herskind et al 1996; Yashin et al 1999; Christensen et al 2006; Willcox et al 2006a; Willcox et al 2006b; Bishop et al 2007*). Indeed, twin studies show that genetic factors contribute to the variation in human lifespan by approximately 25% but in populations with a large number of exceptional survivors, the genetic contribution to lifespan may be much higher (*Gundmundsson et al 2000; Kerber et al 2001; Perls et al 2002; Willcox et al 2006b*).

The number of candidate genes studies, GWAS, meta-analyses and genome scanning about ageing and longevity has increased markedly over the years. The individuate genes are those presumably involved in ageing or longevity. But, nowadays, the nutrient-sensing pathways, i.e. insulin/IGF-1 pathway, are probably the most studied in this field of research.

Nonetheless, many studies have associated antioxidant mechanisms (i.e. superoxide dismutases, SODs), immune-inflammatory responses, lipid metabolism (APOE, APOB, ACE, APOC3) and stress resistance (HSPA1A and HSPA1L) to human longevity and this reflects its multifactorial

influence. To asses this trait and the processes that lead to ageing, specific genes or genome scanning and phenotypic and biochemical effects of gene variants are studied in LLI and centenarians. Indeed, they are the best models for these kind of studies, also because genetic contribution to human longevity has been estimated to be most profound during the late part of life.

Unfortunately, the intrinsic complexity and the heterogeneity among people make studies on ageing and longevity difficult to standardized, also because it is not easy to collect an adequate population in terms of number and information related to previous event happened in life. For this reason, it could be helpful to study model organisms to identify potential candidates and to apply new knowledge or hypothesis to human. Indeed, many studies in model organisms, such as yeast, nematodes, fruit flies and mice, have been conducted demonstrating that a single genetic mutation or a single environmental intervention can modulate lifespan. There are many possible candidate genes for human longevity but, up to now, only one has been shown positive results in different studies and populations: the APOE gene (*Christensen et al 2006*).

The APOE E4 isoform has been linked to elevated cholesterol, CVDs, age-related cognitive decline, and dementia. It is more strongly associated with AD than longevity and other conditions. Homozygosity for the E4 allele confers up to 15-fold risk for AD in whites and 8-fold risk in African Americans compared with the most common ApoE genotype. Thus, ApoE may influence longevity through premature atherosclerosis and age-related diseases (*Murabito et al 2012*).

But a growing body 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 FOXOA3A, 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), ageing and longevity 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.

AKT also regulates the gene expression by modulating the function of several TF. Among these, it can stimulate the NF-kB pathway that is known to be involved in ageing process.

Consequently it could be speculated that a reduction in insulin signaling may reduce the activation of NF-kB thus slow down the transcription of inflammatory genes. As we discussed in our paper (*Balistreri et al 2012*), these kind of genes contribute to immunosenescence, phenomenon also influenced by genetics. The different results obtained in Sicilian centenarians, both males and females, and in subjects with myocardial infarction get further support to the influence of genetics to determine longevity and to the different strategies between males and

females to reach longevity. These strategies are driven by both biological and socio-cultural characteristics and are the reasons for which women live longer that men in developed countries.

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 longevity. Moreover, we agree that inflammatory pathways have a crucial role as well. Indeed, as previously discussed for the Sicilian population, there was an association between SNPs responsible for a low production of inflammatory mediators and longevity.

Unfortunately, one of the limits to the discovery of the common longevity features is probably the gene-gene and gene-environment interaction but many evidences show that genetic factors are involved in longevity in humans and contribute more, after 85 years of age.

In any case, these data clearly suggest that intervention on ageing and longevity should be based both on nutrient pathways and inflammatory network.

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*).

However, although some forms of DR may be beneficial, this severe dietary regimen that induces major health benefits is not a desirable option for most people. Drugs that target nutrient-sensing pathways and mimic the

effects of DR to obtain the health benefits of DR are more realistic, but before they can be considered for chronic administration they require large investments.

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 2010b*).

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*).

Thus these data strengthen the probability that successful ageing may be pharmacologically modulated.

Nowadays, the healthcare costs in many countries are very high because of the increased aged populations and the consequent increase of age-related diseases. Hence, interventions to slow ageing and/or to age successfully are urgently necessary. Indeed, success in increasing longevity in laboratory organisms has shown that ageing is not an immutable process.

It has been calculated that if ageing is slowed for seven years, the age specific risk of death, frailty, and disability will be reduced by about half at every age. People who reach the age of 60 in the future would resemble current 53 year olds, and so on. On the other hand, if ageing is combined with extended years of healthy life, it could also produce unprecedented social, economic, and health dividends. Understanding of the ageing process should have a prominent role in new strategies for extending the health of a population that is highly susceptible to the diseases of ageing.

Thus, investigating ageing and longevity pathophysiology, particularly disentangling low grade inflammation, is likely to provide important clues about how to develop drugs that can slow or delay ageing.

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Desidero ringraziare la Prof. ssa Candore e il Prof. Caruso per avermi guidata, aiutata e spronata durante questi tre anni di dottorato.

Un ulteriore ringraziamento va ai miei colleghi con i quali ho condiviso le “gioie e i dolori” di questa esperienza.

Infine, vorrei ringraziare il mio compagno e la mia famiglia che mi hanno sostenuta moralmente (e non solo) e sopportata durante i miei studi.