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In search of genetic and epigenetic markers of human aging and longevity: a study in the Calabrian population.

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Summary

Aging is a complex process of progressive physical decline that characterizes every biological species and leads to a dramatic reduction of the individual survival probability and, ultimately, to death. The quality and the rate of the aging process are characterized by a considerable variability, which implies noticeable differences in the individual aging phenotype and lifespan. Such variability is due to the interplay of (epi)genetic, environmental, and stochastic factors. In the last decades there has been a growing scientific interest regarding the basis of individual variability in human aging, and several molecular epidemiological studies have been devoted to providing clues about the mechanistic and genetic basis of human aging. This growing interest may be explained by the increasing number of elderly subjects (aged 65 and older) in developed countries over the last 50 years due to the continued improvements in health care. Such a fast increase of these population segments represents a huge problem for the societies in terms of social care and welfare. This problem is strengthened by the high prevalence among these subjects of chronic diseases, cognitive impairment, and other disorders responsible for the increase over time in the functional limitations of older people, and for the decline of their quality of life. Hence, understanding the relationship between disease and aging to increase longevity in a healthy condition is a major goal in medical research.

The overall aim of this PhD project was to identify new genetic (objective 1) and epigenetic (objective 2) determinants of healthy/unhealthy aging and survival up to advanced ages and thereby to improve the understanding of mechanisms modulating the quality of aging and lifespan.

The objective 1 was pursued in four studies where it was investigated the genetic variability of a large number of SNPs in cohorts of individuals comprising: elderly healthy subjects including centenarians (age-range 65-108 years), elderly patients with frailty, and elderly patients affected by late onset Alzheimer diseases (LOAD). Candidate genes were chosen for relevance in signaling pathways affecting nutrient-sensing, energy homeostasis, and in the cellular ability to cope with internal/external insults. The objective 2 was pursued by investigating the expression pattern of a group of specific skeletal muscle miRNAs (myomiRs) in plasma from sarcopenic and non-sarcopenic old individuals, and by analyzing whether changes in the levels of these miRNAs correlated with malnutrition, one of the main causes of the onset of sarcopenia. To achieve this objective, a new cohort of older adults who were living in nursing homes located in the province of Crotone and Cosenza (Calabria region), was recruited.

By applying case-control and perspective survival cohort designs, the following has been observed: (1) sex and age-specific effects of genetic variants, with changes in allele frequency showing either linear or non-linear trajectories, corroborating the observation that SNPs associated with longevity may be either pro-longevity or killing variants, but also deleterious variants neutralized by the protective effect of pro-longevity genes (buffered variant); (2) puzzling association of SNPs with longevity and neurodegenerative disease, with harmful variants for LOAD significantly represented in long-lived subjects. The SNP-SNP interaction analysis suggested this may depend on phenotype-specific synergistic interactions among different genetic markers. Consequently, the possibility of a gene contributing to longevity and/or disease may depend on the aggregated outcome of multiple influences of its variants in the interactome network of cell; (3) a connection among malnutrition, expression of myomiRs and sarcopenia, likely mediated by inflammation and oxidative stress.

Overall, these findings have allowed not only to identify new useful genetic markers of aging and aging-related diseases, but also to confirm and expand knowledge about the complex relationship between healthy/unhealthy aging and longevity.

Sommario

L’invecchiamento è un processo complesso caratterizzato da un progressivo declino fisico che interessa ogni specie vivente e comporta una riduzione drastica della probabilità di sopravvivenza dell’individuo e, infine, la morte. La qualità e il tasso di invecchiamento sono caratterizzati da una considerevole variabilità da individuo a individuo. Questa variabilità è dovuta all’interazione tra fattori (epi)genetici, ambientali e stocastici. Negli ultimi decenni, in campo scientifico si è registrato un interesse crescente nell’identificare i determinanti della variabilità individuale legata all’invecchiamento nell’uomo e diversi studi epidemiologici e molecolari sono stati avviati per fornire evidenze sulle basi meccanicistiche e genetiche dell'invecchiamento umano. Questo interesse crescente può essere spiegato con l’aumento del numero dei soggetti anziani (dai 65 anni in su) negli ultimi 50 anni nei Paesi sviluppati, dovuto anche ai continui miglioramenti nell’assistenza sanitaria. L’incremento così rapido di questa fetta di popolazione rappresenta un enorme problema per le società in termini di assistenza sociale e benessere a causa dell'elevata prevalenza tra questi soggetti di malattie croniche, deterioramento cognitivo e altri disturbi che nelle persone anziane sono responsabili delle limitazioni funzionali e che diventano più severe con l’andare del tempo portando al declino della loro qualità di vita. Quindi, comprendere la relazione tra malattia e invecchiamento al fine di aumentare la longevità in condizioni di salute è uno degli obiettivi principali della ricerca medica.

L'obiettivo generale di questo progetto di dottorato è stato quello di identificare nuovi determinanti genetici (obiettivo 1) ed epigenetici (obiettivo 2) dell’invecchiamento sano / malsano e della sopravvivenza fino ad età avanzate e quindi di migliorare la comprensione dei meccanismi che modulano la qualità dell'invecchiamento e la durata della vita.

L'obiettivo 1 è stato perseguito in quattro studi in cui è stata studiata la variabilità genetica di un gran numero di SNP in coorti di individui comprendenti: soggetti sani anziani, inclusi centenari (fascia di età 65-108 anni), pazienti anziani con fragilità e pazienti anziani affetti da malattie di Alzheimer ad insorgenza tardiva (LOAD). I geni candidati sono stati scelti per la rilevanza nelle vie di segnalazione che influenzano il rilevamento dei nutrienti, l'omeostasi energetica e la capacità cellulare di far fronte ai fattori di stress interni / esterni. L'obiettivo 2 è stato perseguito indagando il pattern di espressione di un gruppo di specifici miRNA del muscolo scheletrico (miomiR) nel plasma di soggetti anziani sarcopenici e non sarcopenici, e analizzando se i cambiamenti nei livelli di questi miRNA fossero correlati alla malnutrizione, una delle principali cause di insorgenza della sarcopenia. Per raggiungere

questo obiettivo, è stata reclutata una nuova coorte di anziani che vivevano in case di cura situate nella provincia di Crotone e Cosenza (regione Calabria).

Utilizzando un approccio di studi caso-controllo e di sopravvivenza prospettica, è stato osservato quanto segue: (1) effetti sesso- ed età-specifici delle varianti genetiche, con cambiamenti nella frequenza degli alleli che mostrano traiettorie lineari o non lineari, avvalorando l'osservazione che gli SNP associati con la longevità possono essere varianti pro-longevità o che sfavoriscono la longevità, ma anche varianti deleterie neutralizzate dall'effetto protettivo dei geni pro-longevità ("buffered variant"); (2) sorprendente associazione di SNP con la longevità e le malattie neurodegenerative, con varianti dannose per LOAD che risultano rappresentate in modo significativo in soggetti di età avanzata. L'analisi dell'interazione SNP-SNP ha suggerito che ciò potrebbe dipendere da interazioni sinergiche fenotipo-specifiche tra diversi marcatori genetici. Di conseguenza, la possibilità che un gene contribuisca alla longevità e / o alla malattia può dipendere dall'effetto combinato di molteplici varianti nella fitta rete di interazioni cellulari; (3) una connessione tra malnutrizione, espressione di miomiR e sarcopenia, probabilmente mediata dall'infiammazione e dallo stress ossidativo.

Nel complesso, questi risultati hanno permesso non solo di identificare nuovi marcatori genetici dell'invecchiamento e delle malattie legate all'invecchiamento, ma anche di confermare ed espandere le conoscenze sulla complessa relazione tra invecchiamento sano / malsano e longevità.

CHAPTER 1

General Introduction

1.1 The demographic trend of aging

Aging is a gradual and inevitable process occurring in organisms. As we age, a physical decline occurs, accompanied by a progressive loss of physiological integrity and the accumulation over the years of systemic damages (López-Otín et al, 2013). The impaired functionality leads to a significant rise in multi-morbidity and to a higher risk of death among older individuals (Park et al, 2013). Nevertheless, some individuals appear someway resistant to causes of death and can reach very old ages in good clinical conditions.

During the last years, the interest of living longer and healthier has notably risen because of the increasing prevalence of old people in worldwide population. Indeed, the fraction of individuals aged more than 60 years is projected to reach 21.1% (> 2 billion) by 2050 (Sander et al, 2015). The *World Population Prospects (2010)* of the United Nations Secretariat has estimated that 5-year-children will be outnumbered by people aged 65 years or older in 2050 (Figure 1).

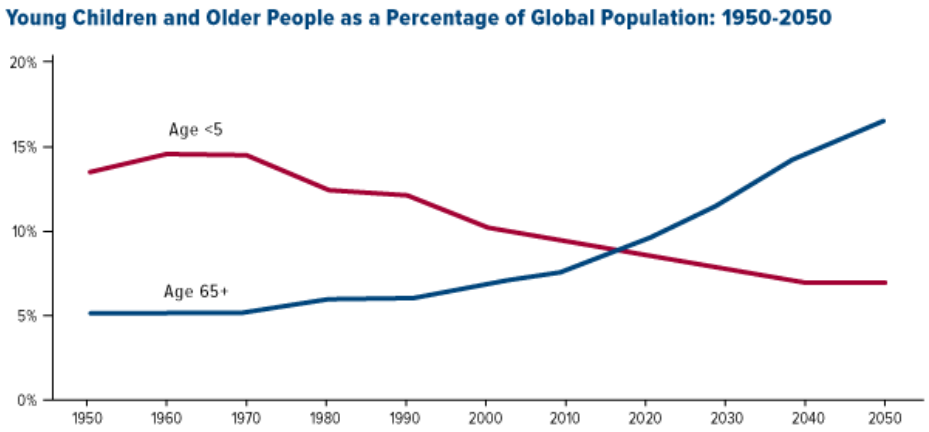

Figure 1. Percentage of young and old population within 1950-2050. (Source: United Nations, *World Population Prospects*: The 2010 Revision, U.S. Global Investors).

Mostly, the gain of years in life expectancy interests developed countries of Western Europe, USA, Japan but in the last few years even developing countries reflects this trend of longer lifespan. In fact, in sub-Saharan Africa, despite the still high fertility rate, the number of people aged over 60 is set to triple from 53 million in 2009 to 150 million by 2050 (Shetty, 2012).

Based on this evidence, during the last two decades, a lot of studies have been carried out in order to better understand the mechanisms occurring during aging with particular focus on biological and molecular factors associated with the variation in aging. Moreover, the World Health Organization (WHO) drew up the Global Strategy on Ageing and Health (2020-2030) in order to plan a concerted

collaboration together governments, civil society, international agencies, professionals, academia and the private sector to improve the lives of older people.

1.2 Aging: why and how

1.2.1. Theories of aging

Over the years several theories have been developed focusing on the different aspects of aging. These theories have not to be considered as mutually exclusive but in combination because they explain mechanisms which operate simultaneously and synergistically during aging.

The main theories give an evolutionary, molecular, cellular and systemic explanation of aging. The first evolutionary theory of aging was proposed in 1952 by Peter Medawar who used the term "senescence" to refer to this process. It suggests that mutations may accumulate in the population if these mutations show beneficial or neutral effects on fitness in early life but are detrimental in later life when selection is inefficient to remove them (Medawar, 1952).

On this idea, George Williams in 1957 formulated the "theory of antagonistic pleiotropy" which posits that some genes that are beneficial early in life may have deleterious effects later (Williams, 1957). An example of genes which show antagonistic pleiotropy is *p53* that protects against cancer by interrupting the abnormal proliferation of cells but this function can be deleterious with aging because it avoids the proliferation of stem cells needed for tissue renewal (Ungewitter et al, 2009).

Another evolutionary explanation of aging is that lifespan is determined by the balance of resources invested in longevity and in reproduction. For this reason, in 1977 Kirkwood proposed "the disposable soma theory" which explains that the somatic organism is maintained only for reproductive fitness, afterward it is disposable. Species, who are unlikely to live long anyway, invest more energy in reproduction than in maintaining somatic cells. On the contrary, humans who can reproduce over a longer period allocate more resources to repair physical damages (Kirkwood, 1977).

The first cellular explanation of aging was provided by Hayflick and Moorhead in the "cellular senescence theory" in 1961. They theorized that the human cells ability to divide is limited to approximately 50-times, after which they simply stop dividing (Hayflick and Moorhead, 1961). Later, this phenomenon was causally linked to the loss of telomeres, repeated DNA sequences at the end of each chromosome. A small amount of this DNA is lost during each cell division and to avoid the accumulation of DNA damages, cells go to the so-called replicative senescence (Aunan et al, 2016). In addition to telomere attrition, several other stresses have been shown to induce a senescent growth

arrest *in vitro*, including DNA lesions, impaired proteostasis, mitochondrial dysfunction and reactive oxygen species (ROS) accumulation.

In this regard, the "Free Radical Theory" was first proposed in 1957 (Harman, 1957). It posits that mitochondrial DNA mutations lead to elevated free radicals by introducing altered enzymes into the electron transport chain and the increased free radicals generate more mitochondrial DNA mutations acting in a vicious cycle and leading to organ failure and senescence (Kauppila et al, 2015). Actually, human aging is accompanied by damaged DNA and oxidized proteins (Krisko et al, 2019). The main hallmarks of cellular senescence are summarized in Figure 2.

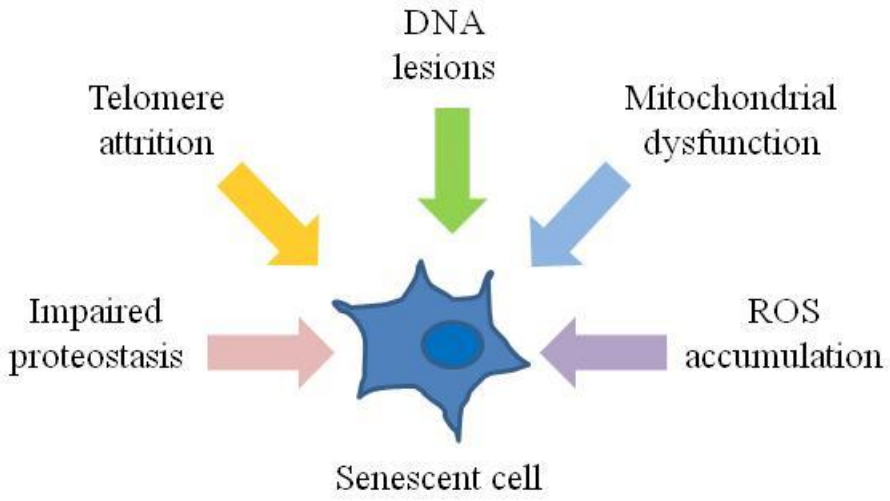

Figure 2. Schematic representation of hallmarks of cellular senescence.

Some theories give a genetic and molecular explanation of aging. The "Gene Regulation Theory" of aging proposes that senescence results from changes occurring in gene expression (Kanungo, 1975). Aging is modulated by a combination of genetic and non-genetic factors. The main genetic factors contributing to the aging phenotype are genes correlated with the maintenance of the cell and of its basic metabolism (Passarino et al, 2016). For example, genes for DNA repair, heat shock response and scavenge of free radicals contribute to longevity and their reduced functionality leads to accelerated cellular aging (Passarino et al, 2016).

Modern theories have been focused on the immune and endocrine changes occurring with aging. In particular, the "Immune theory" suggests that aging is indirectly controlled by a network of cellular and molecular defense mechanisms (Franceschi et al, 2000a) and the "Neuroendocrine theory" posits that aging may be due to alterations in neural and endocrine functions (Fabris, 1991). Neuroendocrine

and immune system tightly interact and an integrated functional decline of these two regulatory systems occurs with aging (Tosato et al, 2007). The neuroendocrine and immune perturbations not only selectively affect neurons and the hormonal cross-communication between various endocrine axes, but also influence the regulation of survival through adaptation to stress (Tosato et al, 2007).

Moreover, a concomitant proinflammatory status, provoked by a continuous antigenic load and stress, characterizes older individuals. Therefore, the so called "Inflamm-aging theory" has been formulated which posits that the persistent inflammatory status occurring in aging may be explained by the macrophage activation during immune and stress response. In fact, macrophages release large amounts of proinflammatory cytokines, neuropeptides, and hormones (Franceschi et al, 2018a).

All the theories reveal that several and important step forward the understanding of the aging process have been done but further studies are still needed and numerous cues solved to clarify to which extent the aging process can be limited or reversed.

1.2.2. Unhealthy aging

The increase in life expectancy associated with the demographic transition has led to an "epidemiological transition" characterized by a high incidence and prevalence of chronic non-communicable diseases such as cardiovascular diseases, diabetes, atherosclerosis, chronic obstructive pulmonary disease, dementia and cancer. Also, the burden of comorbidity and multi-morbidity, the coexistence of several chronic diseases in the same individual, increased dramatically. In Italy, the 56.7% of 65-year-old adults has two or more chronic diseases (Atella et al, 2019). The estimates indicate that the prevalence of four or more comorbidities is higher in the age range 66-80 years (Atella et al, 2019). This condition increases hospitalization and the rate of mortality among elderly, becoming a public health problem because it worsens healthcare cost as it involves a large portion of the population.

The geriatric syndrome known as frailty is a paradigmatic example of a condition correlated with a higher hospitalization and vulnerability to diseases, disabilities, and death in elderly. Frailty is a multidimensional concept and can be defined as a dynamic state that affects an individual with declines in one or more domains, such as physical, cognitive, social, attention or senses. It is characterized by decreased physiological reserves and compromised capacity to maintain homeostasis because of age-related accumulated deficits (Kojima et al, 2019).

The prevalence of frailty increases with age, and it is common especially among subjects aged 80 years or more (Collard et al, 2012). One of the most relevant disabilities related to frailty is sarcopenia, which is characterized by perceived weakness, loss of muscle mass and strength (Liguori et al, 2018). According to the current estimates, 5%–10% of elderly people aged 60–70 years and 11%–50% of those over the age of 80 are facing this disability (Shafiee et al, 2017). Acute and chronic comorbidities can contribute to the development of sarcopenia, leading to a higher risk of adverse outcomes, physical disabilities, compromised quality of life and death, making sarcopenia one of the most common cause of hospitalization, thus imposing a heavy burden on health care spending for the elderly (Ali et al, 2014).

Although age is the main risk factor for the majority of common diseases contributing to disability, the relationship between aging and disease is still rather controversial. For a long time, aging has been considered a disease itself, an old tenet that has been later put apart by the modern medicine that has considered aging and diseases as separate phenomena that could eventually interact but that are essentially different in nature. Their relationship is likely much more complex than previously thought. The most recent hypothesis, the "geroscience hypothesis", posits that aging is the underlying cause leading to the development of many chronic age-related diseases/geriatric syndromes and that aging and age-related diseases share a common set of basic biological mechanisms, including loss of proteostasis, inflammation, dysregulated adaptation to stress and metabolism, damaged macromolecules, stem cell exhaustion and epigenetic alterations (Kennedy et al, 2014). This lead to consider the aging phenotype and the age-related diseases/geriatric syndromes not as separate entities, but rather as the consequences of the same processes which likely proceed at different rates. In other words, the main difference between aging and age-related diseases/geriatric syndromes lies in the intensity and speed of aging cellular and molecular processes, combined with genetic and lifestyle/habit factors. According to this view, these two entities are part of a continuum where precise boundaries do not exist and the two extremes are represented by patients who suffered one or more chronic diseases and show signs of accelerated aging (unhealthy aging), and centenarians who largely avoided or postponed most age-related diseases/geriatric syndromes and are characterized by decelerated aging (healthy aging), respectively. Subjects follow trajectories of accelerated or decelerated aging depending on their genetic, epigenetic and lifestyle background (Franceschi et al, 2018b).

1.2.3. Healthy aging and longevity: the model of centenarians

World's centenarians (age 100+ years) have increased in the last decades. In the nineties, the European prevalence of centenarians was of about 1 per 10,000 whereas nowadays the prevalence rate is around 1 per 5000 (Teixeira et al, 2017). The remarkable growth of centenarians is mainly driven by improved health care, hygiene and healthier life styles and, like in all population segments, and, although a heterogeneity in centenarians' physical and cognitive status exist, depending on their socioeconomic status or their cultural background (Borras et al, 2019), they are considered as a model of successful aging. Centenarians retain independence and capability as well as cognition at higher levels for longer than the general population, together with postponed mortality (Andersen et al, 2012, Arai et al, 2014, Pavlidis et al, 2012).

The "compression of morbidity theory" posits that centenarians live 96% or more of their lives functionally independent and in good health because the period of time in which they experience morbidity is compressed into a small frame time at the end of their lives (Perls, 1997). Evidence which supports this hypothesis shows that a reduction in disability occurs about 2% per year accompanied by a 1% per year decline in mortality during the same period (Pignolo, 2019). Recently, the concept of "resilience" has been suggested to define the organism's ability to recover from stressors that disturb the homeostasis of the system (Kirkland et al, 2016). Centenarians can be considered as an example of resilient individuals. Within this perspective, successful aged subjects do not scape the physiological decline, but its rate is slow enough to be counterbalanced by their resilience (Borras et al, 2020).

Interestingly, a new and growing subpopulation of exceptionally aged individuals has arisen within the centenarian population. Gerontologists have recently defined this new demographic group as semi- (over 105 years old) and supercentenarians (over 110 years old) (Vacante et al, 2012). Centenarians and semi-supercentenarians have been deeply characterized. Centenarians have a good metabolic profile, characterized by preserved glucose tolerance and insulin sensitivity, low serum level of insulin-like growth factor (IGF)-I and IGF-II. Also, the metabolomic signature of centenarians shows peculiar changes in pro- and anti-inflammatory compounds and detoxification (Vacante et al, 2012; Collino et al, 2013).

The general features of exceptional longevity appear to run in families and as a group they have a natural tendency to maintain good health much of their lives. Offspring of centenarians, for instance, have a lower prevalence of age-related diseases, such as cancer, cardiovascular disease, hypertension, and type 2 diabetes. Also, siblings of centenarians have a lower risk to undergo major age-related

diseases when compared to appropriate selected controls from the same population (Terry et al, 2004; Newman et al, 2011). Also, they show beneficial profiles for many metabolic and immune-related parameters (Slagboom et al, 2011).

As survivors at the tail end of an increasingly extended human survival curve, this exceptional population has generated great interest because of its potential to uncover genetic and non-genetic keys to healthy aging and longevity.

1.3 Aging and longevity: Genetic, epigenetic, and environmental factors

The basis of human healthy aging and longevity, and how these phenotypes are achieved, remain one of the main challenges of modern biology and medicine. While studying the genetic basis of aging and longevity can provide important insights on the underlying biological processes, the understanding of lifestyle and environmental factors can provide effective and appropriate strategies to prevent disease and disability and maintain health in the general population.

1.3.1. Genetic contribution to human aging and longevity

Epidemiological evidence for a genetic component to variation in human lifespan mainly comes from twin studies and family studies. Studies in twins reported that the share of the variation in human life span which can be attributed to genetic differences among individuals ranges between 22% and 33%; this genetic contribution is mainly evident after the age of 60 years and seems to increase with age (Barzilai et al, 2012).

A study of 2872 Danish twin pairs born between 1870 and 1900 found that the heritability of extreme longevity was approximately 25% (Herskind et al, 1996). In addition, the heritability of living to at least 100 has been estimated at 0.33 in women and 0.48 in men (Sebastiani et al, 2012a). Subsequently, examples of familial clustering of longevity were reported by Perls and co-workers (Perls et al, 2002a). By analyzing 444 centenarian pedigrees, and by comparing death rates and survival probabilities of siblings of centenarians with data from the same birth cohort, they found that relative survival probability for these siblings increased at old age and was significantly higher when compared with people born in the same birth cohort. What is more, siblings of centenarians had a one-half life-long reduction in risk of death, even up through very old age. These findings were also supported by data obtained from relatives of super-centenarians, where a survival advantage was found for relatives (siblings and offspring) of super-centenarians (Perls et al., 2002a). The Leiden Longevity Study, carried out in the Dutch population, also confirmed the familial clustering of extreme longevity

(Schoenmaker et al, 2006). However, these studies do not distinguish how much of the familial component is genetic or due to environmental factors shared by the members of the family. A study of Hjelmborg and co-workers (2006) showed that having a co-twin surviving to old ages significantly increases the chance of reaching the same old age much more in monozygotic than in dizygotic twins.
Further studies compared the survival function of brothers of centenarians with those estimated for their brothers in law, that is with the men who married their sisters which share the same familiar environment and so may help to distinguish the genetic from the "familiar" effect. This approach showed that the survival advantage was higher for the siblings of long-lived subjects and just partially shared by their brothers in law, despite they shared the same environment for most of their life (Montesanto et al, 2011).
Although these studies clearly support the existence of a genetic component affecting lifespan in humans, especially at advanced ages, the identification of specific genes that robustly associate with longevity has been a challenge (Christensen et al, 2006), and several approaches were carried out to highlight the genetic contribution to long life. Study designs include cross-sectional and prospective studies. Population-based cross-sectional studies evaluate differences between groups of unrelated individuals in categories of increasing age, usually unrelated highly aged individuals (nonagenarians and centenarians) are compared to younger controls. In family- based cross-sectional studies the offspring of long-lived individuals is compared to age-matched controls, either random individuals from the general population or spouses of the offspring of the long-lived individuals. Prospective studies consist of highly or middle-aged individuals (related or unrelated) that are followed over time. Altogether these studies have the potential to provide valuable insights not only into the mechanisms that can drive the biological aging rate and that, then, can positively and negatively influence the risk for age related diseases, but also can explain the variation in lifespan between individuals. To identify loci that influence human longevity or healthy aging or related phenotypes, both linkage and association studies have been carried out. Linkage analysis investigates the co-segregation of markers and a given phenotype in closely related individuals. Linkage studies of long-lived sibships or extended pedigree with exceptionally long-lived individuals have identified several putative longevity linkage. To date, the largest linkage study was done in the multisite European Genetics of Healthy Aging (GEHA) Study, which looked at 2118 European full sib-pairs over 90 years old (Beekman et al, 2013). GEHA found linkage at 4 regions: 14q11.2, 17q12-q22, 19p13.3-p13.11, and 19q13.11-q13.32.

The multiple linkage signals observed in this and other studies indicate the presence of genetic heterogeneity of longevity and healthy aging in human populations.

Association studies investigate the association of specific genetic variants with a given phenotype; for instance, the comparison of allele frequencies between a group of affected individuals (cases) and a group of unaffected individuals (controls). The simplest strategy in association studies is the candidate gene approach, where a set of SNPs flanking a gene which is believed to be involved in healthy/unhealthy aging are tested for their association to longevity in long-lived subjects and in younger geographically matched controls. The underlying hypothesis is that the alleles predisposing to longevity are more frequent in long-lived subjects (such as centenarians) than in the younger subjects, because those carrying alleles predisposing to longevity are likely to survive to the subjects not carrying them and thus, as cohort grows older these alleles become more and more frequent (Perls et al, 2002b). The first studies mainly focused on candidate loci that emerged from model organisms. Studies of inbred lab strains and of natural genetic variants in model organisms such as yeast and worms, flies and mice have clearly implicated many specific genes in the lifespan of these organisms, and have been crucial for the identification of genes that also contribute to human longevity (Taormina et al, 2019). Moreover, many of the candidate genes have been chosen based on their essential roles in pathways correlated to age related diseases (such as inflammation, oxidation, lipid metabolism). Unlike candidate gene studies, which rely on a priori knowledge for gene selection, technological advances have allowed genome-wide association studies (GWAs) to provide an unbiased and hypothesis-free approach to test for associations of common genetic variants across the whole genome. Typically, genotype distributions of 300,000-2,500,000 SNPs are assessed for association with the trait in these studies. To date, numerous GWAS on longevity have been performed which provided additional insight in the drivers of aging and longevity. Among the first studies, Sebastiani et al. in 2012 published a study run on a discovery cohort of more than 800 centenarians and 900 genetically matched controls and two replication cohorts for an additional 300 centenarians and 3200 controls. They generated a list of SNPs potentially associated with exceptional longevity and used the most informative SNPs to build signatures that were able to predict the survival and mortality of the carriers (Sebastiani et al, 2012b).

Thereafter, several other large cohort studies were launched (see Morris et al 2019 for an exhaustive list of such studies).

Using all these approaches, many genes have been associated with longevity or healthy aging phenotypes. Currently, the GenAge (https://genomics.senescence.info/genes/) database lists over 300 human aging-related genes and the LongevityMap (https://genomics.senescence.info/longevity/) database of human genetic association studies contains over 500 entries. Loci exhibiting genome-wide significance for longevity can be retrieved in Morris et al (2019). However, many positive results have not been replicated in different populations. Among the many studied genes, only *APOE* and, to some extent but not always, *FOXO3A* were consistently replicated in different studies (Soerensen et al, 2010, Broer et al, 2015; Silva-Sena et al, 2018). *APOE* encodes the protein apolipoprotein E (ApoE), which mainly functions in lipoprotein-mediated lipid transport, whose importance in late-onset Alzheimer's disease (LOAD) has been firmly established (Belloy et al, 2019). *FOXO3A* encodes the protein forkhead box O3 (FOXO3a), which acts as a transcription factor for many different genes involved in several processes such as oxidative stress and apoptosis (Martins et al, 2016). A meta-analysis on the genetics of human longevity (Deelen et al, 2014) showed genome wide significant association with survival to 90 years of age at two loci: i) one on chromosome 19q13.32, i.e. the previously identified *TOMM40/APOE/APOC1* locus; ii) a new locus on chromosome 5q33. Both loci associate with survival but at opposite directions. Interestingly, the intergenic region on chromosome 5q33.3 is the first GWAS-identified locus promoting human longevity. The *APOE* locus has been recently confirmed to be associated with longevity in many meta-analyses of GWA studies (Sebastiani et al, 2018; Deelen et al, 2019).

Reasons for lack of replication include poorly designed (i.e. differences in phenotype definition) or underpowered (insufficient sample size) studies that will result in false positives that fail to replicate. Also, the discrepancy in the findings may be due to the nature of the variants, which may be rare or even private. Indeed, given the multifactorial nature of longevity and healthy aging, different populations may have different longevity variants due to particular environmental exposures and ancestry-specific genetic difference, and then combining data from populations with different lifestyles and genetic backgrounds, even if well-matched for ethnicity, may hide true association signals (Melzer et al, 2020).

1.3.2. Findings from genetic association studies of human aging and longevity

Although the drawback of replications, the results of longevity studies contributed significantly to our understanding of the underlying mechanisms of aging. Not only these studies allowed to elucidate the biological pathways involved in lifespan determination, which will be discussed later in this chapter,

but also produced important discoveries on the role of genetic risk factors for healthy/unhealthy aging and longevity.

As previously stated, one current view is that aging, age-related diseases, and longevity are part of a continuum without precise boundaries and that they share common mechanistic pillars. Centenarians, which are at one extreme of such continuum, are characterized by two peculiar features: first, the capability to avoid or postpone the major age-related diseases, which suggests that longevity and resistance to diseases are mediated by shared mechanisms, and second a high level of heterogeneity of their phenotype, which suggests that many strategies can be used to become long-lived.

Studies to date highlighted an overlap between the genetics of longevity and the genetics of age-related diseases: among the genes associated to longevity several are disease-related genes (Franceschi et al, 2020; Deelen et al, 2019; Melzer et al, 2020). The prevalent idea is that long-lived individuals are characterized by a reduced prevalence of disease-predisposing variants and an increased prevalence of health-promoting variants. Several studies provided evidence in support of this expectation (Brooks-Wilson, 2013). However, results of many other studies conducted over last decades suggest that the presence of genetic risk factors for major diseases does not always compromise longevity, and that the frequency of many genetic risk variants among centenarians is sometimes like that in younger controls. For instance, the study by Beekman et al. (2010), who compared the distribution of several disease-susceptibility alleles known to be associated with diseases such as cardiovascular disease, cancer and type 2 diabetes between long-lived individuals and younger controls, found a similar distribution of risk alleles in the two groups. Also, it has been reported that many clinically relevant variants for cancer and other diseases are present in genomes of centenarians, suggesting that these variants are compatible with exceptional longevity (Freudenberg-Hua et al, 2014). This is consistent with the results of the study by Sebastiani et al (2012c) showing that the supercentenarian genomes have a comparable number of known disease-associated variants as other genomes.

It has been argued that trade-off-like and conditional effects of genes on phenotypes of health and aging may account for this apparently paradoxical behaviour of the genetic risk factors (Ukraintseva et al, 2016).

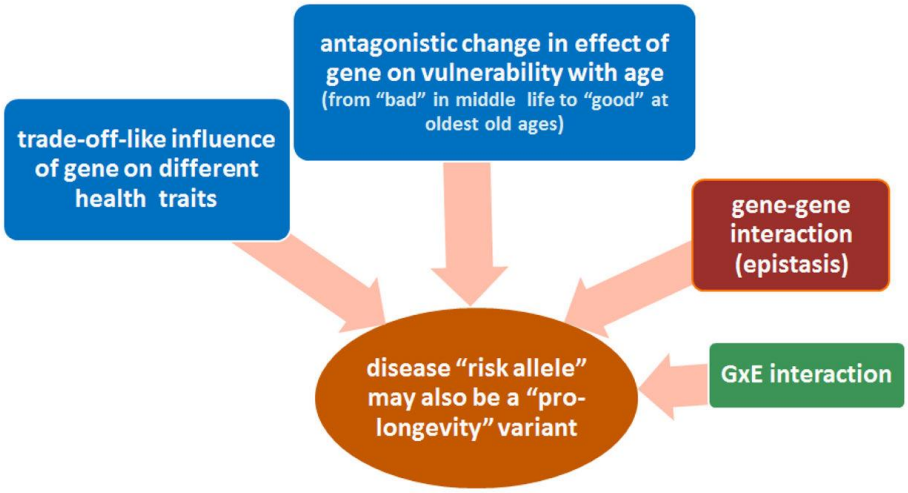

Figure 3. Genetic risk factors for major aging-related diseases do not always compromise longevity (Ukraintseva et al, 2016).

As Figure 3 shows, such effects could be associated to the presence of genetic variants that may antagonistically influence two or more health phenotypes. For instance, they may increase risk of one disease, while reduce risk of another health condition, or improve survival from it. Several studies support the idea that trade-off-like effects can be a common occurrence and contribute to longevity (reviewed in Ukraintseva et al, 2016). A plausible biological mechanism of the genetic trade-offs is likely related to the different roles of the same biological process in the onset and development of many health problems that occur with age. Another reason why disease risk alleles may also be pro-longevity variants is that genes may change their impact on vulnerability to death during one's life. As an example, a genetic variant can be associated with a higher vulnerability to death in middle life but may then become neutral or relatively beneficial at advanced ages (bad" in middle life to a relatively "good" at oldest old ages). This means that such variant will be a risk or a pro-longevity factor depending on age. Literature provides many examples (see references in Ukraintseva et al, 2016). For instance, it has been reported that carriers of the homozygous major allele of a SNP in *NRDE2* gene had negative effect on survival at ages before 80, while positive effect afterwards, as compared with the minor allele carriers (Yashin et al, 2015). It was suggested that long-lived individuals may carry risk alleles for common diseases because the harmful effects of such alleles may be "buffered" by beneficial pro-longevity alleles in other genes (Bergman et al, 2007). These age-specific effects of genes could be the result of antagonistic influence of a gene on diseases and aging-related phenotypes at different ages or even be the product of the changes in internal gene–environment interactions with age. Also, the epistatic interactions can change the effect of an allele on phenotype from detrimental to

beneficial (or vice versa) upon the presence of another allele in another locus. Genetic interactions that significantly contribute to human longevity have been reported in several studies, from which emerges the importance to investigate SNP-SNP and gene-gene epistatic interactions to evaluate the genetic contribution to longevity (Fuku et al, 2017; Dato et al, 2018).

The study of different allele/genotype frequencies in distinct age classes, the so called "gene frequency method", represents a powerful tool to identify the role of each variant according to the trends showed during aging. In particular, the change of allele frequency with age may follow linear (monotonic) trends (the relative prevalence of longevity-promoting or deleterious genotypes being expected to rise and fall monotonically over the life course) or non-monotonic patterns (usually U-shaped patterns), in which allele frequency decreases to a given age but then increases, reflecting trade-offs in their effect at young and old ages.

In addition to age-specific effects, several studies underlined the existence of gender-specific influences of genetic variations on longevity. In fact, besides genes found associated with longevity in both genders, many studies indicated that the association between genetic variants and longevity is often present only for men and not for women, or vice versa (Zeng et al, 2018; Corbo et al, 2013). The different findings in male and female observed in gene/longevity association point out that gender is a major variable in the genetics of longevity and suggest that men and women follow different strategies to reach longevity. To this regard, it has been hypothesized that men invest more than women in genetics to attain longevity, while female are more likely attain longevity because of a more favourable environmental condition and a healthier. This could also explain the generally much higher number of centenarian women with respect to centenarian men, but also the fact that centenarian men are in general in better shape than centenarian women (Franceschi et al, 2000b).

Overall, current evidence suggests that longevity is influenced by many genetic variants with small effect sizes and that such variants may be negative, neutral, or positive, depending on the fine balance between detrimental and beneficial consequences of such variants on several health and aging related traits. This balance may be affected by age, internal and external environments, and by the genetic background.

1.3.3. Epigenetic contribution to aging and longevity

Data are accumulating which show that epigenetics, at the interface between the genome and the environment, may represent an important factor that can impact the rate and the quality of aging, or warn for the onset of age-related diseases. Thus, delineating and understanding the epigenetic changes

that happen during aging is a major ongoing area of study, which may, not only provide better understanding of the mechanisms involved in aging and longevity, but also potentially lead the way to the development of novel therapeutic approaches to delay aging and age-related diseases.

There are different types of epigenetic information encoded within our epigenome, including DNA methylation, posttranslational modifications of the histone proteins, and transcription of noncoding RNAs (ncRNAs) that may be functionally significant for the aging process.

1.3.4. DNA methylation and histone modifications

DNA methylation involves the transfer of a methyl group from S-adenosyl methionine (SAM) to the fifth position of cytosine nucleotides and it plays important role in gene regulation, chromosome stability and genomic imprinting (Xiao et al, 2019). The content of methylated cytosines changes over the human lifetime. Indeed, aging is characterized by global genomic DNA hypomethylation which interests especially repetitive sequences such as Alu (Bollati et al, 2009). A study carried out by Heyn and colleagues showed that the methylation of more than 90% of all CpG sites of human genome was lost in centenarians compared to newborn subjects (Heyn et al, 2012).

However, region-specific hypermethylation is observed in aging leading to a turning off of the involved genes. For example, hypermethylation affects genes involved in vascular aging (*VASH1*), antigen processing (*HLA-E, HLA-F, HLA-B*) (Reynolds et al, 2014), development and growth (*c-myc, c-Fos*) (Akintola et al, 2010), immune response (*EXHX1, IL-10, TSP50, GSTM1, SLC5A5, SPI1, F2R, LMO2, PTPN6, FGFR2, MMP9, MET*) (Yeh et al, 2017).

Based on these evidence, epigenome-wide association studies (EWAS) have identified a set of CpG markers, named "clock CpGs", whose methylation status is measured to predict the age of cells, organs and tissues (Gibson et al, 2019). The Hannum and Horvath clocks are the most recognized models for the age prediction (Hannum et al, 2013; Horvath, 2013). The difference between biological and chronological age is defined epigenetic age acceleration. A positive age acceleration results when the biological age is older than chronological age (Chen et al, 2019).

DNA methylation changes during aging are also influenced by environmental factors such as chemicals, pollutants, drugs, diet, infections and social status (Huidobro et al, 2013). Pregnancy is a period of life particularly sensitive to the exposure to the above factors. Indeed, protein-deficient and energy-enriched diet during pregnancy induce different methylation patterns which are transmitted from mothers to sons thus regulating in offspring long term metabolic processes which contribute to age phenotypes and age-related diseases (Lillycrop et al, 2015).

Moreover, methylation on mitochondrial DNA has a correlation with aging. For instance, in a study carried out on 82 subjects, low methylation levels have been found in 133 CpG sites of mitochondrial DNA and two CpG sites (M1215 and M1313) located within the mitochondrial 12S rRNA gene showed significant hypomethylation with increasing age (Mawlood et al, 2016).

Other epigenetic factors influencing aging are histone modifications. A loss of the control in histone modifications has been observed in elderly (Wang et al, 2018a). Indeed, a disruption of HP1 localization occurs together with the restructuring of heterochromatin, resulting in loss of repression in constitutive heterochromatin loci and gain in facultative heterochromatin (Tsurumi et al, 2012).

In addition, the activity of histone modifying enzymes has species-specific effect on lifespan (Maleszewska et al, 2016). A pivotal role in extending lifespan is attributed to Sirtuins, NAD-dependent deacetylases (Giblin et al, 2014). The Sir2 overexpression increased lifespan in yeast, worms, and flies (Herranz et al, 2010). Moreover, caloric restriction, which is an effective extender of survival, requires Sir2 to produce lifespan extension. In fact, caloric restriction did not have effect on longevity in Sirt1-null mice (Herranz et al, 2010).

In aging, the interplay between DNA methylation and histone modification exists. In fact, some chromatin readers such as methyl-CpG-binding proteins recruit DNA methyl transferases, histone deacetylases and transcriptional factors resulting in a silencing complex (Wang et al, 2018b). A study showed that Methyl-CpG-binding protein 2 increased and SIRT1 expression decreased in senescent endothelial progenitor cells (Wang et al, 2018b). This aspect reveals the complexity of epigenetic influence on aging and longevity which involves many regulators.

1.3.5. Non-coding RNAs

Non-coding RNAs (ncRNAs) are the most recent players in the epigenetics field, influencing seemingly all biological processes. Evidence about the correlation of ncRNAs with age and age-related clinical outcomes has emerged during the last years (Huan et al, 2018). There are several types of ncRNAs such as small nuclear RNA (snRNA), long non-coding RNA (lncRNA), silencing RNA (siRNA) and microRNA (miRNA) (Fiannaca et al, 2017). The latter have been extensively studied underscoring their role in regulating genes expression.

microRNAs are small non-coding single-stranded RNAs of approximately 21-25 nucleotides long which bind to their complementary messenger RNAs (mRNAs) inducing post-transcriptional gene silencing (Hammond, 2015) (Figure 4). Firstly discovered in *Caenorhabditis elegans*, miRNAs are highly conserved in different species.

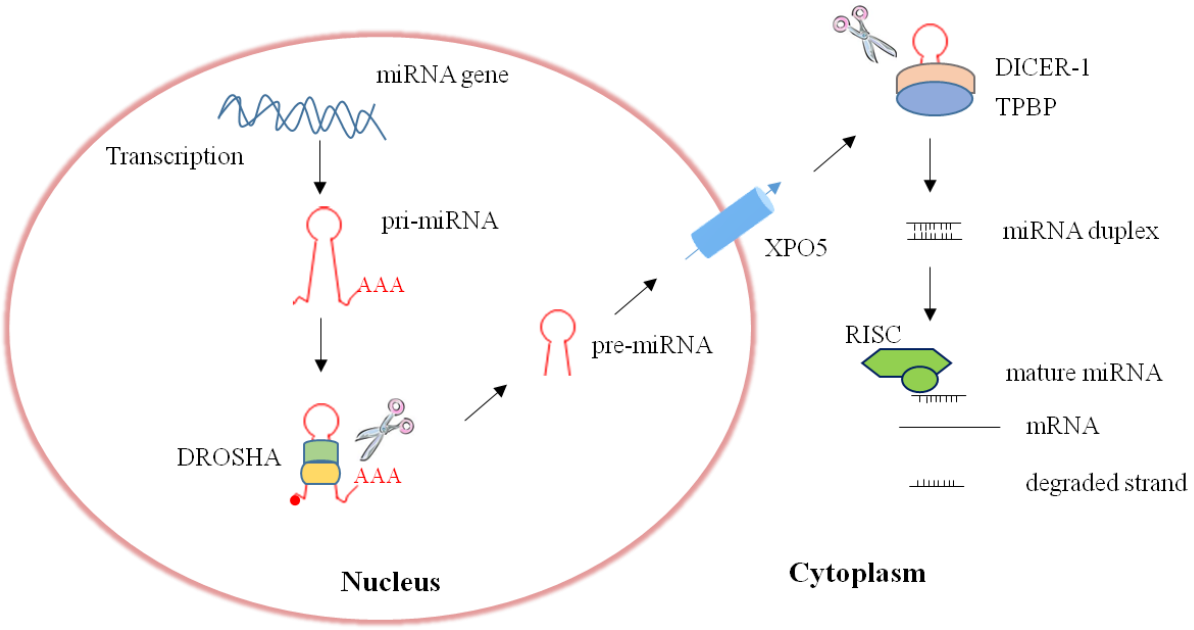

Figure 4. microRNAs biogenesis. Their biogenesis is a complex process which starts from the transcription of a several hundred nucleotides long primary transcripts (pri-miRNAs) by RNA polymerase II with a 5' guanosine cap and a 3' polyadenylated tail. The multiprotein complex cointaining a nuclear RNase III, called Drosha, processes the pri-miRNA in pre-miRNA (70-120 nucleotides). The exportin 5 exports into the cytoplasm the pre-miRNA where is processed into a mature duplex by the RNase III Dicer-1 together with the RNA-binding protein *TPBP*. The strand with perfect base pairing is degraded whereas the second strand associates with Argonaute protein forming the RNA-induced silencing complex (RISC) directed to the target mRNA (Sheng et al, 2018). The target site is mostly recognized by "the seed region" (2-8 nucleotides) at the 5' end of the mature miRNA (Zhang et al, 2017). The main site for miRNA binding is the 3' UTR of a mRNA but recent evidence show that miRNAs can also bind the 5' UTR and the open reading frame (Moretti et al, 2010). The post-transcriptional repression mediated by miRNAs can be played reducing the translational efficiency or decreasing the mRNA levels by degradation. The high complementarity between miRNA and mRNA leads to mRNA degradation instead of translational inhibition (Denzler et al, 2016).

miRNAs are localized both in the intracellular space and outside of the cell. The circulating miRNAs are present in biofluids including blood, plasma, serum, saliva, urine and pleural effusions and contribute to the regulation of cell-cell communication (Sohel et al, 2016).

The first evidence of the role of miRNAs in aging process has been obtained with studies in *C. elegans*. In fact, comparing young, middle-aged and long-lived daf-2 insulin signaling mutants, De Lencastre and colleagues identified 11 miRNAs which changed their expression, mostly decreasing, with age (De Lencastre et al, 2010). The main involved pathways in *C. elegans* were insulin/IGF-1 signaling and cell-cycle checkpoint. Moreover, miR-34 targets the transcription factor DAF-16/FOXO

thus having a role in increasing robustness that means the ability to resist to perturbations, necessary for longevity in the worm (Isik et al, 2016).

These findings prompted to identify age-related miRNAs in different species. In humans, the Baltimore Longitudinal Study of Aging showed that 24 miRNAs were significantly upregulated and 73 were downregulated in the long-lived subgroup (76-92 years) compared with short-lived subjects (58-75 years) (Smith-Vikos et al, 2016). Interestingly, the targets of miRNAs that influence lifespan are genes with a role in aging mechanisms such as insulin signaling, apoptosis, inflammation. For instance, the chronic inflammatory status in aging seems to be characterized by the upregulation of the so-called inflamma-miRs which are involved in toll like receptor and NF-kB pathways in order to restrain the excessive inflammatory response (Rippo et al, 2014).

In addition, miRNAs that target mitochondrial- or nuclear-encoded mitochondrial genes (mitomiRs) have been subjects of studies because mitochondria impairment occurs during aging (Rippo et al, 2014; Rose et al, 2017). For instance, replicative senescent human endothelial cells (HUVECs) show the overexpression of the mitomiRs miR-146a, miR-34a and miR-181a and the consequent downregulation of their target Bcl-2 in comparison with young cells. Bcl-2 has anti-apoptotic and anti-oxidant functions, and therefore its downregulation through the action of miRNAs causes increased oxidative stress and impaired functions (Rippo et al, 2014). In addition, the mitomiRs seem to be involved in NF-kB and insulin pathways, as observed for inflamma-miRs. Some mitomiRs can reduce insulin secretion by targeting genes that encode for mitochondrial transporters (Baradan et al, 2017).

The impaired post-transcriptional inhibition mediated by miRNAs and their aberrant expression patterns contribute to the development of age-related diseases. Since the first detected functions of miRNAs concerned cell division, apoptosis, intracellular signaling and cellular metabolism, the early stages of the research in human diseases have been carried out to evaluate the role of miRNAs in cancer. Evidence have shown that some miRNAs are differentially expressed during various cancer progression stages, thus they are helpful to define diagnoses and for prognosis assessments (Paranjape et al, 2009).

Altered miRNA functions contribute to cardiovascular diseases with a major incidence in aged individuals. The so-called athero-miRs alert the altered blood flow occurring in the formation of the atherosclerotic plaque, therefore they can be used for the prediction of atherosclerosis (Kumar et al, 2014).

Interestingly, a small group of muscle-specific miRNAs, termed myomiRs, has been identified. MyomiRs include miR-1, miR-206, miR-208a, miR-208b, miR-133a, miR-133b, miR-486 and miR-499 (Brown et al, 2015). These myomiRs are both expressed in cardiac and skeletal muscle except miR-206 that is specific for skeletal muscle and miR-208a that is specific for heart. Their functions have been firstly discovered in knocked-out mice experiments (McCarthy, 2011) and include myoblast proliferation, differentiation, regeneration, cell fate regulation, chromatin remodeling and oxidative stress control (Horak et al, 2016). MyomiRs can be released by cells during muscle wasting and detected in body fluids (Izzotti et al, 2018). Their stability in body fluids allows them to be potentially used as non-invasive biomarkers of muscle damage together with other circulating miRNAs involved in muscle functionality.

The future perspectives may be addressed to the better detection of miRNA sets in aged tissues to understand more efficiently their way of action.

1.3.6. Environmental contribution to aging and longevity

The entirety of life-course environmental exposures and the lifestyle factors to which an individual is subjected, from conception till death is defined as 'exposome' (Wild, 2005). The exposome is a variable and dynamic entity which evolves throughout the lifetime and synergistically offends the organism with an accumulation of damages determining a multisystem loss of reserve and function. By affecting the internal biological pathways and signaling mechanisms such injuries result in an increased susceptibility to chronic diseases and an acceleration of the aging process (Misra, 2020). During the last decades, several evidence documented the potential impact of environmental factors on aging and lifespan, in terms of both intrinsic, such as oxidative stress, and extrinsic factors, like toxins, nicotine consumption, xenobiotics such as pharmaceuticals, pesticides and insecticides, food additives, synthetic and industrial chemicals, and microbial compounds which constitute a huge part of the human chemical exposome and are also associated with chronic human diseases (Sorrentino et al, 2014). As a consequence of the interactions existing among exposome and endogenous proteome, epigenome, genome and metabolome (Go et al, 2014), the organismal ability to positively interact with the exposome is an adaptive response deriving by an optimal balance of such a network (Jones, 2015). A sedentary lifestyle and an unhealthy diet are also considered among the external environmental influences affecting the human health. In fact, the lack of physical activity, impacting on skeletal muscle decline, contributes to disabilities and increased mortality at advanced ages (Cartee et al, 2016). Moreover, starting from the third decade of life (Booth et al, 2011), a sedentary life accelerates

the maximal oxygen consumption decline, leading to accumulated mitochondrial deficits (Gouspillou et al, 2014). On the contrary, exercise has a positive anti-aging impact: in fact, while endurance exercise seems to mitigate physiological changes, maintaining insulin sensitivity and functionality of mitochondrial respiratory chain, resistance exercise helps to gain muscle strength (Cartee et al, 2016). At a multisystem level, a regular physical activity, especially aerobic and resistance training, is able to maintaining cardio-respiratory fitness and cognitive function, boosting metabolic activity, and leading to improved functional independence (Rebelo-Marques et al, 2018). Furthermore, a regular aerobic exercise can influence gene transcription through DNA methylation, activating for example genes which encode for telomere-stabilizing proteins (Rebelo-Marques et al, 2018). It is known that telomere stability is protective for muscle injuries and a positive correlation exists between the telomere length and the number of satellite cells in skeletal muscles of older adults, leading to muscle regeneration and preservation (Werner et al, 2009). Studies on centenarian cohorts confirmed the importance of exercise on lifespan, suggesting that also at very old age the levels of physical activities correlate to health status and independence (Venturelli et al, 2012).

In combination with physical activity, a healthy diet is beneficial and essential for anti-aging effects, in particular a balanced diet, which supplies all the necessary nutrients for health. However, individuals may respond differently to specific diet interventions: for instance, people with hepatic insulin resistance respond better to a low-fat diet, whereas those with insulin resistance in the muscles respond better to a Mediterranean diet (Blanco-Rojo et al, 2016).

In animal models, including rats, mice, fish, flies, worms and yeast, a general beneficial effect was attributed to a calorie-restriction diet, mainly correlated to the reduced metabolic rate and oxidative stress, improved insulin sensitivity, and changed neuroendocrine and sympathetic nervous system function (Heilbronn et al, 2003). Caloric restriction is referred to a dietary intervention with an overall 20–40% reduction of total caloric intake. The rhesus monkeys are the closest model organism to humans in which caloric restriction has been experimentally tested in a controlled environment, showing both the delay of age-related diseases and improved lifespan (Colman et al, 2009). In humans, observational studies reported health benefits for prolonged caloric restriction, in the context of high-quality diets. In particular, a randomized study on non-obese subjects showed that a two year 25%-caloric restriction cause a reduction of inflammatory markers and cardiometabolic risk factors (Lee et al, 2016). However, caloric restriction was also associated with adverse effects, like reduced bone mineral density (Villareal et al, 2006). The most accepted hypothesis is that caloric restriction extends

lifespan by down-regulating the insulin and mTOR (mammalian target of rapamycin) pathways, leading to a balanced protein synthesis (Kenyon, 2010). A correct caloric restricted and balanced diet can also avoid the decrease of beneficial species in the gut microbiota, which otherwise result in gut-related diseases (Kumar et al, 2016). Microbiota with its metabolic activity may be altered by the progressive toxin and chemical exposures (Claus et al, 2016). The importance of gut microbiota on lifespan was suggested by a comparative analysis of the gut microbiota composition and fecal metabolites between a group of centenarians and a younger control group of subjects (25–45 years), which highlighted that some bacteria like *Ruminococcaceae* can be considered as a common signature of longevity in different populations (Tuikhar et al, 2019).

1.4 Converging Pathways in the Regulation of Lifespan

In humans, as previously discussed, huge studies showed that searching for longevity genes cannot discard genes involved in age-related diseases. Aging and age-related diseases are, indeed, characterized by a multifactorial etiology and share some biochemical-molecular mechanisms that contribute to their manifestation. Thus, to gain more insights into the relationship between healthy and unhealthy aging is necessary to move from single gene to the fine analysis of the integrated network of genes, looking for the key hubs and connectors laying at the crossroad between health and disease, successful and unsuccessful aging.

Among the cellular mechanisms influencing health and shared with common diseases, experts suggest seven master regulators, represented by adaptation to stress, proteostasis, stem cell exhaustion, metabolic derangement, macromolecular damage, epigenetic modifications, and inflammation (Kennedy et al, 2014). The possible combinations of these events occurring during aging make it particularly challenging to reveal the interplay and hierarchical order of these mechanisms as well as to study their consequences at a molecular level. In the past few decades, various model organisms (invertebrate and vertebrate) have been extensively exploited to investigate the mechanisms of aging, risk factors and the so called “longevity pathways”.

In particular, studies on model organisms revealed the nutrient-sensing pathways as determinant hub paths of longevity. Most notably, the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway, mammalian target of rapamycin (mTOR), and adenosine monophosphate-activated protein kinase (AMPK) play important roles in regulating aging and lifespan (Templeman et al, 2018).

The insulin/IGF-1 promotes the phosphorylation and the exclusion from the nucleus of Forkhead box O (FOXO) proteins, thus suppressing FOXO-dependent genes transcription which are involved in

glucose metabolism, apoptosis, DNA repair, proliferation and cell differentiation (de Almeida et al, 2017). Increased longevity is associated with lower levels of IGF-1 because of reduced cell proliferation, better oxygen consumption and increased reliance on fatty acid oxidation which can have beneficial effects on lifespan (Pan et al, 2017). Interestingly, centenarians show a switch in comparison to younger adults because they have increased IGF-1 plasma levels which contribute to the improved insulin action. In fact, long-lived subjects show a higher insulin sensitivity, a better preservation of beta-cell function, a greater glucose uptake and lower insulin-mediated lipolysis with a consequent decline in plasma free fatty acid and triglyceride concentrations (Vitale et al, 2019).

Moreover, insulin/IGF-1 pathway and the cellular energy sensor AMPK send signals to mTOR which is a serine/threonine kinase composed by mTOR complex1 (mTORC1) and mTORC2. mTORC1 plays an integral role in regulating growth and metabolism by regulating several downstream targets involved in protein translation, lipid and pyrimidine synthesis, mitochondrial metabolism and decreased autophagy (Pan et al, 2017). mTORC2 modulates the metabolic and spatial control through the actin cytoskeleton regulation (Stallone et al, 2019).

Dysregulated mTOR signaling is linked to various detrimental processes occurring with aging such as impaired proteostasis, increased elongation speed which causes incorrect proteins translation, inhibition of autophagy, promoted mitochondrial dysfunction and cellular senescence (Papadopoli et al, 2019). The suppression of mTOR leads to reduced secretion of pro-inflammatory cytokines, improved intestinal and hematopoietic stem cell self-renewal, increased ability to respond to stress and preserved autophagic machinery which avoids cellular damages accumulation (Pan et al, 2017). Therefore, the quelling of mTOR prolongs lifespan and delays the onset of age-related diseases (Papadopoli et al, 2019).

Beyond of the important role of the nutrient-sensing signaling, the relevance to aging of new nutrient hubs, often called metabolic sensors, is now attracting attention, and has become the focus of intense investigation. In particular, increased AMPK activation resulted to improve lifespan by maintaining mitochondrial homeostasis and coordinating with peroxisomes to increase fatty acid oxidation (Weir et al, 2017). Among the metabolic sensors, Sirtuins (SIRTs), a highly conserved group of NAD^+ dependent protein deacetylases that responds to high NAD^+/NADH ratios, are considered master regulators of eukaryotic aging based on pioneering work on yeast lifespan (Pan et al, 2017). In fact, overexpression of SIRTs extend lifespan regulating mitochondrial biogenesis, inflammatory suppression and genomic stability (Wątroba et al, 2016). Among the seven types of sirtuins, SIRT1

seems to protect from cardiovascular failure, certain forms of cancer and neurodegenerative diseases (Pan et al, 2017).

Despite a tendency to conceptualize and investigate IIS, mTOR, and AMPK as separate signaling pathways, it is important to consider that there is cross-talk between them, and some amount of overlap in their downstream targets. For instance, nutrient depletion induces the suppression of mTOR and the activation of AMPK which, in turn, increases NAD^+ levels culminating in sirtuins activity. Then, sirtuins can deacetylate FOXO, changing the insulin/IGF-1-mediated transcription (Pan et al, 2017) (Figure 5).

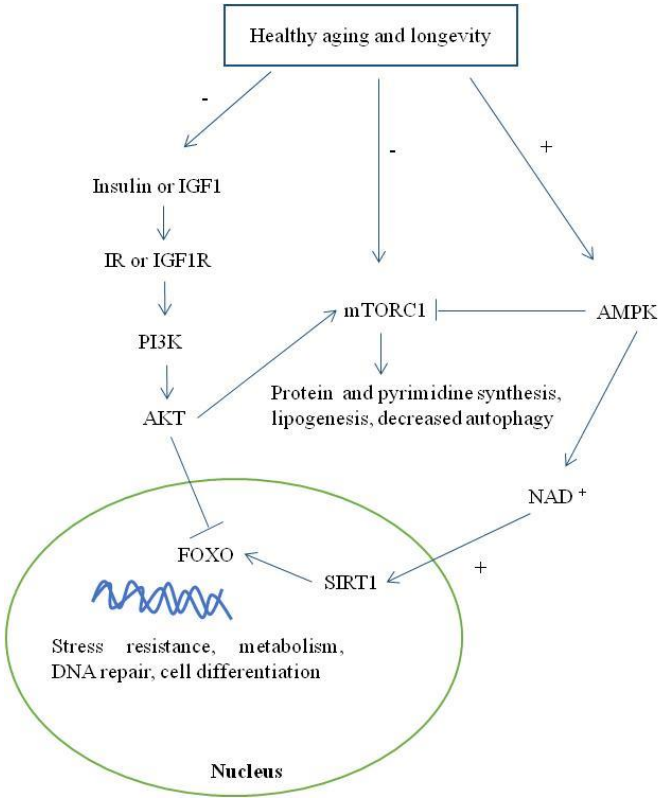

Figure 5: Interactions between Insulin signaling, mTORC1, AMPK and Sirtuins in healthy aging and longevity. IR and IGF1R (insulin-receptor and insulin-like growth factor 1 receptor); PIP3 (phosphoinositide-3-kinase); AKT (protein kinase B); NAD (Nicotinamide adenine dinucleotide).

This synergy suggests that a global comprehension is needed when dissecting the processes involved in regulating aging and longevity. Also, to gain a more understanding of the system rather than focusing on individual factors, the multi-level integration of omics data should be applied. In recent years, the availability of very large datasets of biological data (genetic variants, epigenomics, RNA expression, and metabolomics) from high-throughput technologies provided valuable tools to

investigate the aging process at the molecular level. Thus, after decades of reductionist studies, network and integrated omics data analysis have begun to target the aging process at a system level, promising to revolutionize the identification of aging biomarkers and the development of strategies to improve health in old age.

1.5 Aim and outline of the thesis

The identification of the determinants of human aging and survival has the potential to provide insight in the mechanisms that may delay or escape the onset of age-related diseases. Since knowledge of such mechanisms may shed a light on the complexity underlying the complex relationship between healthy/unhealthy aging and longevity, the overall aim of this PhD project was to contribute to the understanding of the determinants of human lifespan. To fulfil this aim, the specific objectives are the following:

Specific objective 1: To identify common genetic variants associated with healthy/unhealthy aging and longevity;

Specific objective 2: To identify epigenetic markers of healthy/unhealthy aging.

In this thesis, we have chosen to report the studies and researches as published in relevant scientific journals, in order to offer the reader a further scientific validation of the data contained therein.
Thereofore, four chapters (**Chapters 2-3-4-5)** are addressed to Objective 1 studies**,** in which we investigated the association of aging and related phenotypes and longevity with common genetic variation in candidate genes, chosen for their crucial role in the control of cellular homeostasis and in the individual ability to properly cope with xenobiotic insults. SNP selection was driven by a tag-SNP approach prioritizing those most likely to be of functional relevance. The study population included individuals enrolled within the framework of several recruitment campaigns carried out for monitoring the quality of aging in Calabria from 2002. The age-range of the subjects was between 65 and over 105 years old. All subjects underwent a multidimensional geriatric assessment to properly evaluate the health status and functional reserve. In addition, common clinical haematological tests were performed. Survival data were prospectively collected. On this sample, two approaches have been

used: cross-sectional study which analyses data from a representative subset of population at a specific point in time and longitudinal study, using 10-year overall survival (death from any cause) data.

In study I **(Chapter 2)** we analysed 35 variants in 23 xenobiotic-metabolizing enzyme (*XME*) genes, including phase I, phase II metabolizing enzymes and phase III transporters, which contribute to metabolism, biotransformation, elimination and/or detoxification of endogenous and xenobiotic compounds to which individuals are exposed during their lifetime. Thus, by modulating the dynamic and the complex biological response to the exposome, *XME* genes may influence the individual chance of becoming long-lived.

In study II (**Chapter 3**) we examined the genetic variability of inositol polyphosphate multikinase (*IPMK*) gene, which is emerging as a multifunctional gene encoding a protein with different activities. By mediating synthesis of inositol pyrophosphate, which are secondary messengers regulating many aspects of cell physiology, and by acting non-catalytically via protein–protein interactions, IPMK regulate a wide spectrum of metabolic pathways (from nutrient-sensing to oxidative stress and telomere maintenance) involved in the aging process. Therefore, in study II **(Chapter 3)**, by using single-SNP and haplotype-based approaches, we tested the hypothesis that the genetic variability of *IPMK* may contribute to aging, aging-related phenotypes and survival to very old age.

The study III **(Chapter 4)** was motivated by results from study II in which specific *IPMK* haplotypes were found to affect lifespan in women and by results of a previous study by Crocco et al (2016) that associated SNPs of the inositol hexakisphosphate kinase 3 (*IP6K3*) gene, which encodes for an essential enzyme for inositol pyrophosphates synthesis, with the susceptibility to late onset Alzheimer's disease (LOAD), one of the major neurodegenerative diseases of aging. Based on these findings, in study III we checked for potential cross-phenotype associations by testing whether the genetic variability of *IP6K3*, previously associated with LOAD can affect the ability to live longer and whether the genetic variability of *IPMK*, previously correlated to longevity, can affect the susceptibility to LOAD.

As previously stated, frailty is a condition of elderly characterized by increased vulnerability resulting from aging-associated decline in reserve and function, determining an increased risk for poor health outcomes and mortality. Over the past years, there has been an increasing concern about this syndrome because of the growing number of elderly people in the general population. The Longevity-Associated Variant (LAV) of the bactericidal/permeability-increasing fold-containing family B member 4 (*BPIFB4*) was found significantly enriched in long-living individuals and also it has been found to

correct endothelial dysfunction, one of the mechanisms underlying frailty, in aging mice (Villa et al, 2015). Thus, in study IV (**Chapter 5**) we investigated the hypothesis that *BPIFB4* haplotypes segregate with the frailty phenotype in human.

The second research objective is addressed in study V **(Chapter 6)**. This study was motivated by the growing interest in the identification of biomarkers able to capture key features of aging including physical and cognitive capabilities. Sarcopenia, the biological substrate for the development of physical frailty, severely limits the physical capabilities of older people and is considered one of the major health issues in aging population. Therefore, determination of biomarkers of sarcopenia could be useful to progress the understanding of this condition and to develop new diagnostic tools and effective treatments. miRNAs are emerging as potential biomarkers in different disease conditions. Thus, in study V **(Chapter 6)** we investigated whether muscle-specific miRNA (myomiRs) are differentially expressed in plasma from sarcopenic and non-sarcopenic old individuals. And also, since malnutrition is one of the main causes of the onset of sarcopenia, we analysed whether changes in the levels of these miRNAs correlate with nutritional status.

To fulfil this objective, we recruited a new cohort of older adults who were living in nursing homes, located in the province of Crotone and Cosenza (Calabria region). At recruitment, eligible and consenting participants were subjected to a multidimensional geriatric evaluation through a structured questionnaire administered during an interview with a trained operator. Assessments included measurement of physical and cognitive performance, and collection of information on health status, life style and demographics. In addition, peripheral blood samples of all participants were collected, processed for the purpose of clinical and genetic analysis. Enrolled subjects were categorized as sarcopenic and non-sarcopenic based on measuring muscle mass (Skeletal Muscle Index), muscle strength (Hand Grip Strength), and physical performance (Gait Speed) according to the revised criteria suggested by the European Working Group on Sarcopenia in Older People (EWGSOP2).

In the last chapter (**Chapter 7**) a general discussion of the PhD thesis and the conclusive remarks are presented.

References

1. Akintola AD, Parrish AR. Gene-Specific Hypermethylation in Aging. Epigenetics of Aging. 2010; 29-39
2. Ali S, Garcia JM. Sarcopenia, cachexia and aging: diagnosis, mechanisms and therapeutic options - a mini-review. Gerontology. 2014;60(4):294–305.
3. Andersen SL, Sebastiani P, Dworkis DA, et al. Health span approximates life span among many supercentenarians: compression of morbidity at the approximate limit of life span. J Gerontol A Biol Sci Med Sci. 2012;67(4):395-405.
4. Arai Y, Inagaki H, Takayama M, et al. Physical Independence and Mortality at the Extreme Limit of Life Span: Supercentenarians Study in Japan. J Gerontol A. 2014;69(4):486–494.
5. Atella V, Piano Mortari A, Kopinska J, et al. Trends in age-related disease burden and healthcare utilization. Aging Cell. 2019;18(1):e12861.
6. Aunan JR, Watson MM, Hagland HR, et al. Molecular and biological hallmarks of ageing. BJS 2016; 103: e29-e46.
7. Baradan R, Hollander JM, Das S. Mitochondrial miRNAs in diabetes: just the tip of the iceberg. Canadian journal of physiology and pharmacology. 2017;95:1156-1162.
8. Barzilai N, Guarente L, Kirkwood TB, et al. The place of genetics in ageing research. Genetics. 2012;13: 589-592.
9. Beekman M, Nederstigt C, Suchiman HED, et al. Genome-wide association study (GWAS)-identified disease risk alleles do not compromise human longevity. Proc. Natl. Acad. Sci. 2010;107:18046–18049.
10. Beekman M, Blanché H, Perola M, et al. Genome-wide linkage analysis for human longevity: Genetics of Healthy Aging Study. Aging Cell. 2013;12:184-93.
11. Belloy ME, Napolioni V, Greicius MD. A Quarter Century of APOE and Alzheimer's Disease: Progress to Date and the Path Forward. Neuron. 2019;101(5):820-838.
12. Bergman A, Atzmon G, Ye K, et al. Buffering mechanisms in aging: a systems approach toward uncovering the genetic component of aging. PLoS computational biology. 2007;3:e170.
13. Blanco-Rojo R, Alcala-Diaz JF, Wopereis S, et al. The insulin resistance phenotype (muscle or liver) interacts with the type of diet to determine changes in disposition index after 2 years of intervention: the CORDIOPREV-DIAB randomized clinical trial. Diabetologia. 2016;59:67–76.
14. Bollati V1, Schwartz J, Wright R, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev. 2009;130(4):234-9.
15. Booth FW, Laye MJ, Roberts MD. Lifetime sedentary living accelerates some aspects of secondary aging. J Appl Physiol (1985). 2011;111(5):1497-504.
16. Borras C, Ingles M, Mas-Bargues C, et al. Centenarians: An excellent example of resilience for successful ageing. Mech Ageing Dev. 2020;186:111199.
17. Broer L, Buchman AS, Deelen J, et al. GWAS of longevity in CHARGE consortium confirms APOE and FOXO3 candidacy. J Gerontol A Biol Sci Med Sci. 2015;70(1):110-8.

18. Brooks-Wilson AR. Genetics of healthy aging and longevity. Hum Genet. 2013;132(12):1323-1338.
19. Brown DM, Goljanek-Whysall K. microRNAs: Modulators of the underlying pathophysiology of sarcopenia? Ageing Research Reviews. 2015;24(B): 263-273.
20. Cartee GD, Hepple RT, Bamman MM, et al. Exercise Promotes Healthy Aging of Skeletal Muscle. Cell Metab. 2016;23(6):1034–1047.
21. Chen M, Wong EM, Nguyen TL, et al. DNA methylation-based biological age, genome-wide average DNA methylation, and conventional breast cancer risk factors. Sci Rep. 2019;9: 15055.
22. Christensen K, Johnson TE, Vaupel JW. The quest for genetic determinants of human longevity: challenges and insights. Nat Rev Genet. 2006;7(6):436-448.
23. Claus SP, Guillou H, Ellero-Simatos S. The gut microbiota: a major player in the toxicity of environmental pollutants? [published correction appears in NPJ Biofilms Microbiomes. 2017 Jun 22;3:17001]. NPJ Biofilms Microbiomes. 2016;2:16003.
24. Collard RM, Boter H, Schoevers RA, et al. Prevalence of frailty in community-dwelling older persons: a systematic review. J Am Geriatr Soc. 2012; 60(8):1487-92.
25. Collino S, Montoliu I, Martin FP, et al. Metabolic signatures of extreme longevity in northern Italian centenarians reveal a complex remodeling of lipids, amino acids, and gut microbiota metabolism. PLoS One. 2013;8(3):e56564.
26. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009;325(5937):201–4.
27. Corbo RM, Pinto A, Scacchi R. Gender-specific association between FSHR and PPARG common variants and human longevity. Rejuvenation Res. 2013;16(1):21-7.
28. Crocco P, Saiardi A, Wilson MS, et al. Contribution of polymorphic variation of inositol hexakisphosphate kinase 3 (IP6K3) gene promoter to the susceptibility to late onset Alzheimer's disease. Biochim Biophys Acta. 2016;1862(9):1766-73.
29. Dato S, Soerensen M, De Rango F, et al. The genetic component of human longevity: new insights from the analysis of pathway-based SNP-SNP interactions. Aging Cell. 2018;17: e12755.
30. de Almeida AJPO, Ribeiro TP, de Medeiros IA. Aging: Molecular Pathways and Implications on the Cardiovascular System. Oxid Med Cell Longev. 2017;2017:7941563.
31. De Lencastre A, Pincus Z, Zhou K, et al. MicroRNAs both promote and antagonize longevity in *C. elegans*. Current biology. 2010;20(24):2159-2168.
32. Deelen J, Beekman M, Uh HW, et al. Genome-wide association meta-analysis of human longevity identifies a novel locus conferring survival beyond 90 years of age. Hum Mol Genet. 2014;23(16):4420-32.
33. Deelen J, Evans DS, Arking DE, et al. A meta-analysis of genome-wide association studies identifies multiple longevity genes. Nat Commun. 2019;10:3669.
34. Denzler R, McGeary SE, Title AC, et al. Impact of MicroRNA Levels, Target-Site Complementarity, and Cooperativity on Competing Endogenous RNA-Regulated Gene Expression. Molecular Cell. 2016;64(3):565-579.

35. Fabris N. Neuroendocrine-immune interactions: a theoretical approach to aging. Arch Gerontol Geriatr. 1991;12(2-3):219-30.
36. Fiannaca A, La Rosa M, La Paglia L, et al. nRC: non-coding RNA Classifier based on structural features. BioData Mining. 2017;10: 27.
37. Franceschi C, Bonafe M, Valensin S, et al. Inflamm-aging–An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000a;908:244–54.
38. Franceschi C, Motta L, Valensin S, et al. Do men and women follow different trajectories to reach extreme longevity? Italian Multicenter Study on Centenarians (IMUSCE). Aging (Milano). 2000b;12(2):77-84.
39. Franceschi C, Garagnani P, Parini P, et al. Inflammaging: a new immune-metabolic viewpoint for age-related diseases. Nat Rev Endocrinol. 2018a;14(10):576-590.
40. Franceschi C, Garagnani P, Morsiani C, et al. The Continuum of Aging and Age-Related Diseases: Common Mechanisms but Different Rates. Front Med (Lausanne). 2018b;5:61.
41. Franceschi C, Garagnani P, Olivieri F, et al. The contextualized genetics of human longevity: JACC focus seminar. J Am Coll Cardiol. 2020; 75:968–79.
42. Freudenberg-Hua Y, Freudenberg J, Vacic V, et al. Disease variants in genomes of 44 centenarians. Molecular genetics & genomic medicine. 2014;2:438–450.
43. Fuku N, Díaz-Peña R, Arai Y, et al. Epistasis, physical capacity-related genes and exceptional longevity: FNDC5 gene interactions with candidate genes FOXOA3 and APOE. BMC Genomics. 2017;18(suppl 8):803.
44. Giblin W, Skinner ME, Lombard DB. Sirtuins: guardians of mammalian healthspan. Trends Genet. 2014;30(7):271-86.
45. Gibson J, Russ TC, Clarke T-K, et al. A meta-analysis of genome-wide association studies of epigenetic age acceleration. PLoS Genet. 2019;15(11):e1008104.
46. Go YM, Jones DP. Redox biology: interface of the exposome with the proteome, epigenome and genome. Redox Biol. 2014;2:358-360.
47. Gouspillou G, Bourdel-Marchasson I, Rouland R, et al. Mitochondrial energetics is impaired in vivo in aged skeletal muscle. Aging Cell. 2014;13(1):39-48.
48. Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev. 2015;87:3-14.
49. Hannum G, Guinney J, Zhao L, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular cell. 2013;49:359–367.
50. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1957;2:298-300.
51. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp. Cell Res. 1961;25:585–621.
52. Heilbronn LK, Ravussin E. Calorie restriction and aging: review of the literature and implications for studies in humans, The American Journal of Clinical Nutrition. 2003;78(3):361–369.
53. Herranz D, Serrano M. SIRT1: recent lessons from mouse models. Nat Rev Cancer. 2010;10(12):819–823.

54. Herskind AM, McGue M, Holm NV, et al. The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870-1900. Hum Genet. 1996;97(3):319-323.
55. Heyn H1, Li N, Ferreira HJ, et al. Distinct DNA methylomes of newborns and centenarians. Proc Natl Acad Sci U S A. 2012;109(26):10522-7.
56. Horak M, Novak J, Bienertova-Vasku J. Muscle-specific microRNAs in skeletal muscle development. Dev Biol. 2016;410(1):1-13.
57. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013;14: R115.
58. Huan T, Chen G, Liu C, et al. Age-associated microRNA expression in human peripheral blood is associated with all-cause mortality and age-related traits. Aging Cell. 2018;17(1):e12687.
59. Huidobro C, Fernandez AF, Fraga MF. Aging epigenetics: causes and consequences. Mol Aspects Med. 2013;34(4):765-81.
60. Isik M, Blackwell TK, Berezikov E. MicroRNA mir-34 provides robustness to environmental stress response via the DAF-16 network in *C. elegans*. Scientific Reports. 2016;6:36766.
61. Izzotti A, Longobardi M, La Maestra S, et al. Release of MicroRNAs into Body Fluids from Ten Organs of Mice Exposed to Cigarette Smoke. Theranostics. 2018;8(8):2147–2160.
62. Jones DP. Redox theory of aging. Redox Biol. 2015;5:71-79.
63. Kanungo MS. A model for ageing. J. Theor. Biol. 1975; 53(2):253-261.
64. Kauppila JH, Stewart JB. Mitochondrial DNA: Radically free of free-radical driven mutations. Biochim Biophys Acta. 2015;1847(11):1354-61.
65. Kennedy BK, Berger SL, Brunet A, et al. Geroscience: linking aging to chronic disease. Cell. 2014;159(4):709–13.
66. Kenyon CJ. The genetics of ageing. Nature. 2010;464(7288):504-12.
67. Kirkland JL, Stout MB, Sierra F. Resilience in aging mice. J. Gerontol. A Biol. Sci. Med. Sci. 2016;71:1407–1414.
68. Kirkwood T. Evolution of ageing. Nature. 1977;270:301–304.
69. Kojima G, Liljas AEM, Iliffe S. Frailty syndrome: implications and challenges for health care policy. Risk Manag Healthc Policy. 2019;12:23–30.
70. Krisko A, Radman M. Protein damage, ageing and age-related diseases. Open Biol. 2019;9:180249.
71. Kumar S, Kim CW, Simmons RD, et al. Role of flow-sensitive microRNAs in endothelial dysfunction and atherosclerosis – "Mechanosensitive Athero-miRs." Arteriosclerosis, thrombosis, and vascular biology. 2014;34(10):2206-2216.
72. Kumar M, Babaei P, Ji B, et al. Human gut microbiota and healthy aging: Recent developments and future prospective. Nutr Healthy Aging. 2016;4(1):3–16.
73. Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. F1000Res. 2016;5:F1000 Faculty Rev-117.
74. Liguori I, Russo G, Aran L, et al. Sarcopenia: assessment of disease burden and strategies to improve outcomes. Clin Interv Aging. 2018;13:913–927.
75. Lillycrop KA, Burdge GC. Maternal diet as a modifier of offspring epigenetics. J Dev Orig Health Dis. 2015;6(2):88-95.

76. López-Otín C, Blasco MA, Partridge L, et al. The hallmarks of aging. Cell. 2013;153(6):1194–1217.
77. Maleszewska M, Mawer JSP, Tessarz P. Histone Modifications in Ageing and Lifespan Regulation. Curr Mol Bio Rep. 2016;2:26–35.
78. Martins R, Lithgow GJ, Link W. Long live FOXO: unraveling the role of FOXO proteins in aging and longevity. Aging Cell. 2016;15(2):196-207.
79. Mawlood SK, Dennany L, Watson N, et al. Quantification of global mitochondrial DNA methylation levels and inverse correlation with age at two CpG sites. Aging (Albany NY). 2016;8(4):636–641.
80. McCarthy JJ. The MyomiR network in skeletal muscle plasticity. Exerc Sport Sci Rev. 2011;39(3):150-154.
81. Medawar PB. An unsolved problem of Biology. London: H.K Lewis. 1952.
82. Melzer D, Pilling LC, Ferrucci L. The genetics of human ageing. Nat Rev Genet. 2020;21(2):88-101.
83. Misra BB. The Chemical Exposome of Human Aging. Front. Genet. 2020.
84. Montesanto A, Latorre V, Giordano M, et al. The genetic component of human longevity: analysis of the survival advantage of parents and siblings of Italian nonagenarians. Eur J Hum Genet. 2011;19:882-6.
85. Moretti F, Thermann R, Hentze MW. Mechanism of translational regulation by miR-2 from sites in the 5′ untranslated region or the open reading frame. RNA. 2010;16(12):2493-2502.
86. Morris BJ, Willcox BJ, Donlon TA. Genetic and epigenetic regulation of human aging and longevity. Biochim Biophys Acta Mol Basis Dis. 2019;1865(7):1718-1744.
87. Newman AB, Glynn NW, Taylor CA, et al. Health and function of participants in the Long Life Family Study: A comparison with other cohorts. Aging (Albany NY). 2011;3: 63-76.
88. Pan H, Finkel T. Key proteins and pathways that regulate lifespan. J Biol Chem. 2017;292(16):6452-6460.
89. Papadopoli D, Boulay K, Kazak L, et al. mTOR as a central regulator of lifespan and aging. F1000Res. 2019;8:F1000.
90. Paranjape T, Slack FJ, Weidhaas JB. MicroRNAs: tools for cancer diagnostics. Gut. 2009;58(11):1546–1554.
91. Park DC, Yeo SG. Aging. Korean J Audiol. 2013;17(2):39–44.
92. Passarino G, De Rango F, Montesanto A. Human longevity: Genetics or Lifestyle? It takes two to tango. Immun Ageing. 2016;13:12.
93. Pavlidis N, Stanta G, Audisio RA. Cancer prevalence and mortality in centenarians: a systematic review. Crit Rev Oncol Hematol. 2012;83(1):145-52.
94. Perls TT. Centenarians prove the compression of morbidity hypothesis, but what about the rest of us who are genetically less fortunate? Med. Hypotheses. 1997;49:405–407.
95. Perls TT, Wilmoth J, Levenson R, et al. Life-long sustained mortality advantage of siblings of centenarians. Proc. Natl. Acad. Sci. USA 2002a; 99:8442 – 47.
96. Perls T, Kunkel LM, Puca AA. The genetics of exceptional human longevity. J Mol Neurosci. 2002b;19(1-2):233-8.

97. Pignolo RJ. Exceptional human longevity. Mayo Clin. Proc. 2019;94:110–124.
98. Rebelo-Marques A, De Sousa Lages A, Andrade R, et al. Aging Hallmarks: The Benefits of Physical Exercise. Front Endocrinol (Lausanne). 2018;9:258.
99. Reynolds LM, Taylor JR, Ding J, et al. Age-related variations in the methylome associated with gene expression in human monocytes and T cells. Nat Commun. 2014;5:5366.
100. Rippo MR, Olivieri F, Monsurrò V, et al. MitomiRs in human inflamm-aging: A hypothesis involving miR-181a, miR-34a and miR-146a. Experimental Gerontology. 2014;56:154-163.
101. Rose G, Santoro A, Salvioli S. Mitochondria and mitochondria-induced signalling molecules as longevity determinants. Mechanisms of Ageing and Development. 2017;165:115-128.
102. Sander M, Oxlund B, Jespersen A, et al. The challenges of human population ageing. Age Ageing. 2015;44(2):185–187.
103. Schoenmaker M, de Craen AJ, de Meijer PH, et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. Eur J Hum Genet 2006;14:79-84.
104. Sebastiani P, Perls TT. The genetics of extreme longevity: lessons from the new England centenarian study. Front. Genet. 2012a;3:277.
105. Sebastiani P, Solovieff N, Dewan AT, et al. Genetic signatures of exceptional longevity in humans. PLoS One. 2012b;7(1):e29848.
106. Sebastiani P, Riva A, Montano M, et al. Whole genome sequences of a male and female supercentenarian, ages greater than 114 years. Front Genet. 2012c;2:90.
107. Sebastiani P, Gurinovich A, Nygaard M, et al. APOE alleles and extreme human longevity. J Gerontol A Biol Sci Med Sci. 2018;74:44.
108. Shafiee G, Keshtkar A, Soltani A, et al. Prevalence of sarcopenia in the world: a systematic review and meta- analysis of general population studies. J Diabetes Metab Disord. 2017;16:21.
109. Sheng P, Fields C, Aadland K, et al. Dicer cleaves 5'-extended microRNA precursors originating from RNA polymerase II transcription start sites. Nucleic Acids Res. 2018;46(11):5737–5752.
110. Shetty P. Grey matter: ageing in developing countries. The Lancet. 2012;379(9823):1285-1287.
111. Silva-Sena GG, Camporez D, Santos LRD, et al. An association study of FOXO3 variant and longevity. Genet Mol Biol. 2018;41(2):386-396.
112. Slagboom PE, Beekman M, Passtoors WM, et al. Genomics of human longevity. Philos Trans R Soc Lond B Biol Sci. 2011;366: 35-42.
113. Smith-Vikos T, Liu Z, Parsons C, et al. A serum miRNA profile of human longevity: findings from the Baltimore Longitudinal Study of Aging (BLSA). Aging (Albany NY). 2016;8(11):2971-2983.
114. Soerensen M, Dato S, Christensen K, et al. Replication of an association of variation in the FOXO3A gene with human longevity using both case-control and longitudinal data. Aging Cell. 2010;9(6):1010-7.
115. Sohel MH. Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges. Achievements in the Life Sciences. 2016;10(2):175-186.

116. Sorrentino JA, Sanoff HK, Sharpless NE. Defining the toxicology of aging. Trends Mol Med. 2014;20(7):375-84.
117. Stallone G, Infante B, Prisciandaro C, et al. mTOR and Aging: An Old Fashioned Dress. Int J Mol Sci. 2019;20(11):2774.
118. Taormina G, Ferrante F, Vieni S, et al. Longevity: Lesson from Model Organisms. Genes (Basel). 2019;10(7):518.
119. Teixeira L, Araújo L, Jopp D, et al. Centenarians in Europe. Maturitas. 2017;104:90–95.
120. Templeman NM, Murphy CT. Regulation of reproduction and longevity by nutrient-sensing pathways. J Cell Biol. 2018;217(1):93-106.
121. Terry DF, Wilcox MA, McCormick MA, et al. Lower all-cause, cardiovascular, and cancer mortality in centenarians' offspring. J Am Geriatr Soc. 2004;52: 2074-6.
122. Tosato M, Zamboni V, Ferrini A, et al. The aging process and potential interventions to extend life expectancy. Clin Interv Aging. 2007;2(3):401-412.
123. Tsurumi A, Li WX. Global heterochromatin loss: a unifying theory of aging? Epigenetics. 2012;7:680–8.
124. Tuikhar N, Keisam S, Labala RK, et al. Comparative analysis of the gut microbiota in centenarians and young adults shows a common signature across genotypically non-related populations. Mech Ageing Dev. 2019;179:23-35.
125. Ukraintseva S, Yashin A, Arbeev K, et al. Puzzling role of genetic risk factors in human longevity: "risk alleles" as pro-longevity variants. Biogerontology. 2016;17(1):109-127.
126. Ungewitter E, Scrable H. Antagonistic pleiotropy and p53. Mech Ageing Dev. 2009;130(1-2):10–17.
127. Vacante M, D'Agata V, Motta M, et al. Centenarians and supercentenarians: a black swan. Emerging social, medical and surgical problems. BMC Surg. 2012;12 Suppl 1(Suppl 1):S36.
128. vB Hjelmborg J, Iachine I, Skytthe A, et al. Genetic influence on human lifespan and longevity. Hum Genet. 2006;119(3):312-21.
129. Venturelli M, Schena F, Richardson RS. The role of exercise capacity in the health and longevity of centenarians. Maturitas. 2012;73(2):115-120.
130. Villareal DT, Fontana L, Weiss EP, et al. Bone mineral density response to caloric restriction-induced weight loss or exercise-induced weight loss: a randomized controlled trial. Arch Intern Med. 2006;166(22):2502–10.
131. Vitale G, Pellegrino G, Vollery M, et al. ROLE of IGF-1 System in the Modulation of Longevity: Controversies and New Insights From a Centenarians' Perspective. Front Endocrinol (Lausanne). 2019;10:27.
132. Wang C, Wang F, Li Z, et al. MeCP2-mediated epigenetic regulation in senescent endothelial progenitor cells. Stem Cell Res Ther. 2018b;9(1):87.
133. Wang Y, Yuan Q, Xie L. Histone Modifications in Aging: The Underlying Mechanisms and Implications. Curr Stem Cell Res Ther. 2018a;13(2):125-135.
134. Wątroba M, Szukiewicz D. The role of sirtuins in aging and age-related diseases. Adv Med Sci. 2016;61(1):52-62.

135. Weir HJ, Yao P, Huynh FK, et al. Dietary Restriction and AMPK Increase Lifespan via Mitochondrial Network and Peroxisome Remodeling. Cell Metab. 2017;26(6):884-896.e5.
136. Werner C, Fürster T, Widmann T, et al. Physical exercise prevents cellular senescence in circulating leukocytes and in the vessel wall. Circulation. 2009;120(24):2438-47.
137. Wild CP. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol Biomarkers Prev. 2005;14(8):1847-50.
138. Williams GC. "Pleiotropy, natural selection, and the evolution of senescence". Evolution. 1957;11(4): 398–411.
139. Xiao FH, Wang HT, Kong QP. Dynamic DNA Methylation During Aging: A "Prophet" of Age-Related Outcomes. Front Genet. 2019;10:107.
140. Yashin AI, Wu D, Arbeeva LS, et al. Genetics of aging, health, and survival: dynamic regulation of human longevity related traits. Front Genet. 2015;6:122.
141. Yeh SH, Liu CL, Chang RC, et al. Aging-dependent DNA hypermethylation and gene expression of GSTM1 involved in T cell differentiation. Oncotarget. 2017;8(30):48591–48602.
142. Zeng Y, Nie C, Min J, et al. Sex Differences in Genetic Associations With Longevity. JAMA Netw Open. 2018;1(4):e181670.
143. Zhang F, Wang D. The Pattern of microRNA Binding Site Distribution. Genes. 2017;8(11): 296.

CHAPTER 2

Inter-Individual Variability in Xenobiotic-Metabolizing Enzymes: Implications for Human Aging and Longevity

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1. Introduction

Aging is a complex phenotype responding to a plethora of drivers in which genetic, behavioral, and environmental factors interact with each other. This can be conceptualized in terms of exposome—that is, the totality of exposures to which an individual is subjected throughout a lifetime and how those exposures affect health [1].

The exposome basically includes a wide variety of toxic or potentially harmful compounds of exogenous (environmental pollutants, dietary compounds, drugs) or endogenous (metabolic by-products such as those resulting from inflammation or lipid peroxidation, oxidative stress, infections, gut flora) origin and related biological responses during the life course [2].

The individual ability to properly cope with xenobiotic stress can influence susceptibility to diseases and, thus, the quality and the rate of aging, phenotypes that certainly result from the cumulative experiences over lifespan. Additionally, in all the different theories proposed to explain the aging process, a common denominator remains the progressive decline of the capacity to deal with environmental stressors to which the human body is constantly exposed.

In this scenario, a crucial role can be played by the coordinated activity of cellular mechanisms evolved for reducing the toxicity of endogenous and xenobiotic compounds to which humans are exposed. These mechanisms comprehend a broad range of reactions of detoxification that make harmful compounds less toxic, more hydrophilic, and easier to be excreted. The main effectors of these mechanisms are a large number of enzymes and transporters, collectively referred to as xenobiotic-metabolizing enzymes (XMEs) or drug metabolizing enzymes (DMEs). This process occurs in three phases. Phase I enzymes, such as cytochrome P450s (CYPs), carboxylesterases, and flavin monooxygenases, add reactive groups to the toxin; in phase II, glutathione S-transferases (GST), UDP-glucuronosyltransferases (UGT), catechol-*O*-methyltransferases (COMT), and *N*-acetyltransferases (NAT) conjugate water-soluble groups onto the molecule; in phase III, ATP-binding cassette (ABC) transporter proteins facilitate the export of the conjugate out of cells as well as the import and the efflux of a broad range of substrates [3].

With aging, there is a decline in the ability to mount a robust response to xenobiotic insults. This is somewhat attributed to the age-related reduction in liver mass, which can result in reduced metabolism rates and in the decreased kidney and liver blood flows, which can result in reduced excretion and elimination of xenobiotic and its metabolites [4]. In addition, a reduction in the activity of phase I and II enzymes and the consequent fall in biotransformation capacity have been reported by several

authors in both old animals and humans [5–7]. As aging is characterized by an increased prevalence of chronic conditions that require the use of multiple medications, these changes have particular relevance from a clinical point of view, affecting drug effectiveness and toxicity [8]. Moreover, transcriptional profiling has revealed the up-regulation of xenobiotic-metabolizing genes in long-lived mutants across diverse model organisms [9–11], suggesting that the individual ability to modulate xenobiotic responses may either lead to increased risk of diseases and death or favor longevity.

It is also known that the activity of XME proteins is affected by the variability of the corresponding genes, whose polymorphisms can account for the inter-individual variability in both xenobiotic response/toxicity and disease predisposition. In this regard, significant associations of alleles in these genes (especially in phase II genes) with many forms of cancer [12,13] or coronary heart disease [14] were found. Moreover, in testing a sample of individuals of different ages, Ketelslegers et al. [15] found that the prevalence of risk alleles in XME genes decreases with age, suggesting that individuals carrying a higher number of risk alleles show a higher risk of morbidity and mortality for chronic diseases.

Based on all the above, we reasoned that genetic variants of XME genes might affect the chance to live a long life. In order to test this hypothesis, we screened a set of 35 SNPs in 23 XME genes and their association with aging and survival in a cohort of 1112 individuals aged 20–108 years, performing both case-control and prospective cohort analyses.

2. Materials and Methods

Study Population

The initial dataset included 1112 unrelated individuals (497 men and 615 women) whose ages ranged from 20 to 108 years. All subjects were born in Calabria (Southern Italy), and their Calabrian ancestry was ascertained up to the third generation. Samples were collected within the framework of several and appropriate recruitment campaigns carried out for monitoring the quality of aging in the whole of Calabria, as previously reported [16]. In brief, younger subjects were recruited from students and staff of the University of Calabria; elderly subjects were from people visiting thermal baths, the Academy of the Elderly, or contacted through general physicians. Very old subjects were selected through the population registers and then contacted and invited to join the study. Old and very old subjects underwent a multidimensional geriatric assessment with the aim of collecting clinical history, anthropometric measures, cognitive functioning, functional activity, and physical performance.

White blood cells (WBC) from blood buffy coats were used as sources of DNA, while plasma/sera were used for routine laboratory analyses.

For the analyses, the sample was divided in three specific age classes based on two age thresholds, 65 and 89 years, after which a significant negative change in the slope of the survival curve of the Italian population occurs [17]. Thus, subjects were classified as younger adults (age class S1, 20–64 years; *n* = 330), elderly (age class S2, 65–89 years; *n* = 433), and very old subjects (age class S3, ≥90 years; *n* = 349).

For subjects of the 65- to 89-year-old group, vital status was traced after a mean follow-up time of approximately 10 years through the population registers of the municipalities where the respondents lived.

Ethic Statement

The study was approved by the Ethical committee of the University of Calabria (Rende, Italy, on 9 September 2004). All the subjects provided written informed consent in accordance with institutional requirements and the Declaration of Helsinki principles.

Cognitive and Physical Assessments

Cognitive status was assessed by age- and education-adjusted Mini Mental State Examination (MMSE) [18]. The score ranges from 0 to 30, and a score of 23 points or less is usually considered to indicate cognitive impairment. Hand grip (HG) strength was evaluated by using a handheld dynamometer (SMEDLEY's dynamometer TTM) while the subject was sitting with the arm close to the body. Three consecutive measurements were performed with the stronger hand, and the maximum value was used for data analysis. The performance of activities of daily living (ADL) (bathing, dressing, toileting, transfer from bed to chair, and feeding) was assessed using a modification of the Katz Index [19]. Scores were dichotomized as 1 if the subject was able to perform every activity and as 0 otherwise. Depressive symptoms were assessed using the 15-item Geriatric Depression Scale (GDS) [20]. Subjects with GDS scores greater than or equal to 5 were considered to be affected by depressive symptom.

SNPs Selection and Primer Design for iPLEX TM Assay

A panel of 35 candidate polymorphisms was selected based on known functional consequence (exonic, regulatory regions) or prior associations with disease risk, pathology, or drug response. SNPs were chosen from 23 genes involved in detoxification related pathways of xenobiotic substances.

Supplementary Table S1 reports the complete list of assayed SNPs and their basic features. In summary, we selected 6 SNPs in 5 genes of phase I, 11 SNPs in 7 genes of phase II, and 13 SNPs in 7 genes of phase III. Four non-canonical XME genes (indicated as others in Table S1) and relative polymorphisms (4 SNPs) were selected because they have been deeply studied in relation to drug response, and thus likely affect the risk or the clinical evolution of several diseases. All SNPs have a reported minor allele frequency of >0.05 in Europeans. For each polymorphism, PCR and extension primers were designed using Sequenom MassARRAY Assay Designer 3.0 software (Sequenom, San Diego, CA, USA), resulting in a 22 plex and a 13 plex.

Sequenom Mass Spectrometry Genotyping

First, 2 µL of genomic DNA (5 ng/uL) were PCR-amplified in a 5 µL reaction containing 0.8 µL HPLC grade water, 0.5 µL of 10 × PCR buffer with 20 mM $MgCl_2$, 0.4 µL of 25 mM $MgCl_2$, 0.1 µL of 25 mM dNTP mix, 1 µL of 0.5 µM primer mix, and 0.2 µL Sequenom PCR enzyme. PCR conditions were: an initial cycle at 94 °C for 2 min, 45 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final step at 72 °C for 5 min.

Unincorporated dNTPs in the amplification products were dephosphorylated by adding 2 µL of the shrimp alkaline phosphatase (SAP, Sequenom, San Diego, CA, USA)) mix consisting of 1.53 µL of HPLC grade water, 0.17 µL of SAP buffer, and 0.3 µL (0.5 U) of SAP enzyme (Sequenom). Each reaction was incubated at 37 °C for 40 min, and SAP was then heat inactivated at 85 °C for 5 min.

Following SAP treatment, a single base pair extension reaction was performed using Sequenom's iPLEX Gold chemistry, where 2 µL of the iPLEX reaction mix was added to the samples. The reaction mix consisted of 0.62 µL of HPLC grade water, 0.2 µL of iPlex buffer, 0. 2µL of iPlex terminator mix, 0.94 µL of primer mix, and 0.04 µL of iPlex enzyme. Thermal cycling conditions included an initial cycle at 94 °C for 30 s; 40 cycles at 94 °C for 5 s, [52 °C for 5 s and 80 °C for 5 s (repeat 5 times per cycle)]; and a final step at 72 °C for 3 min. The samples were then resin treated and spotted on a SpectroCHIP using the MassARRAY nanodispenser (Sequenom) and analyzed using the MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF) (Sequenom). Genotypes were assigned in real time using the MassARRAY SpectroTYPER RT v3.4 software (Sequenom) based on the mass peaks present. All results were manually inspected using the MassARRAY TyperAnalyzer v3.3 software (Sequenom).

Quality Control

After genotyping, samples were subjected to a battery of quality control (QC) tests. At sample level, subjects with a proportion of missing genotypes higher than 10% were dropped from the analysis. At SNP level, SNPs were excluded if they had a significant deviation from Hardy–Weinberg equilibrium (HWE, $p < 0.05$) in the younger subgroups, a missing frequency (MiF) higher than 20%, and a minor allele frequency (MAF) lower than 1%. See Table S1 for details.

Statistical Analysis

For each SNP, allele and genotype frequencies were estimated by gene counting from the observed genotypes. HWE was tested by Fisher's exact test. Logistic regression models were used to evaluate the effect of genotypes (independent variables) on the probability of belonging to different age groups (dependent variable). Differences between age groups were tested by comparing two of them at once. Genetic data were coded with respect to a dominant, a recessive, and an additive model of inheritance. Then, for each SNP, the most likely genetic model was estimated on the basis of minimum level of statistical significance (Wald test *p*-value). In such models, sex was used as a covariate. To capture sex-dependent effects of the analyzed genetic variants, an additional interaction term was also included. Finally, to test whether combinations of SNPs might better differentiate between the different age groups, a multivariate model including the associated SNPs was also fitted. The Nagelkerke index was then used to compare the obtained models.

Linear and logistic regression models were applied to estimate the impact of genetic variability on parameters of cognitive (MMSE, GDS) and physical (HG, ADL) performance, including age, gender, and height as covariates. Continuous and categorical variables were compared by using the independent samples *t*-test and the X^2 test as appropriate. For evaluating if the effect of the polymorphisms on longevity phenotype also affected the survival patterns of the different genotypes, we performed a longitudinal study after 10 years from the baseline visit. Univariate survival analysis was carried out by the Kaplan–Meier approach, and survival curves were compared by log-rank test. Subjects were considered as censored if they were alive after the follow-up time, and this time was used as censoring data in the survival analyses. Moreover, hazard ratios (HR) and 95% CI were estimated by Cox proportional hazard models using age and gender as confounder variables.

Because this was a hypothesis driven study, a level of significance *p*-value = 0.05 was considered for each association test without Bonferroni post hoc correction for multiple comparisons.

Statistical analyses were performed using SNPassoc and surv packages of R (http://www.R-project.org/).

3. Results

After quality control checks, there were genotyping data on 27 SNPs in a cohort of 981 individuals aged 20–108 years. Demographic characteristics for the study cohort according to age groups defined in the Materials and Methods section are presented in Table 1.

Table 1. Demographic characteristics for the analyzed cohort according to age group membership.

	Age Class S1	**Age Class S2**	**Age Class S3**
n	287	379	315
Male%	43.6	49.1	35.9
Age Range (years)	20–64	65–89	90–108
(mean, SE)	42.7 (0.89)	73.5 (0.31)	97.8 (0.75)
ADL (% Disabled)	-	43.0	69.5
GDS (% Depressed)	-	32.3	26.2
HG (Kg; mean, SE)	-	22.2 (0.62)	13.1 (0.43)
MMSE <23 (%)	-	9.5	66.5

ADL, activity daily living; GDS, Geriatric Depression Scale; HG, hand grip; MMSE, Mini Mental State Examination; SE, standard error.

Four SNPs demonstrated a nominally significant association (p-value < 0.05) in at least one comparison (see Table 2).

Table 2. Multinomial logistic analysis for univariate genetic associations.

		*Comparison 1 (Age class 2 *vs* Age class 1) 65-89 years vs <65 years		Comparison 2 (Age class 3 *vs* Age class 1) 90+ vs <65 years		Comparison 3 (Age class 3 *vs* Age class 2) 90+ vs 65-89 years	
	Gene	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
rs3745274-G/T	*CYP2B6*	0.97 (0.67-1.40)	0.88	0.54 (0.37-0.80)	0.002	0.56 (0.39-0.80)	0.005
rs776746-G/A	*CYP3A5*	1.20 (0.65-2.22)	0.57	1.97 (1.08-3.56)	0.022	1.66 (0.99-2.79)	0.054
rs4680-G/A	*COMT*	1.67 (1.10-2.54)	0.016	2.43 (1.58-3.73)	<0.001	1.47 (1.10-2.15)	0.046
rs2273697-G/A	*ABCC2*	0.87 (0.61-1.23)	0.44	1.26 (0.89-1.79)	0.18	1.45 (1.03-2.05)	0.030

* In each comparison, the youngest group was considered as the reference category. For both comparisons 1 and 2 (both using the youngest group as reference category), odd ratios (ORs) were obtained directly from the equations included in the models; for comparison 3 (90+ years vs. <65 years), ORs were obtained by difference of equations included in the models.

Two of them (rs3745274-G/T and rs776746-G/A) belonged to genes for phase I enzymes (*CYP2B6* and *CYP3A5*, respectively), one (rs4680-G/A) was within the phase II *COMT* gene, and one (rs2273697-G/A) was within the phase III *ABCC2* gene. The best-fitting genetic model for three of them was dominant, while rs4680-G/A best fit a recessive genetic model. As Figure 1 shows, these variants had different gene frequency trajectories over the three examined age intervals.

We found a significant decrease in the proportion of carriers of the *CYP2B6* rs3745274-T allele in the oldest sample (S3 group) with respect to the youngest S1 and S2 [odds ratio (OR) = 0.547 CI 95% 0.373–0.803, p-value = 0.002 for comparison 2; OR = 0.563 CI 95% 0.395–0.803, p-value = 0.005 for comparison 3), consistent with a detrimental effect of this allele on longevity (Table 2 and Figure 1A). An opposite effect was observed for rs776746, being carriers of the A allele significantly overrepresented in the S3 group as compared to S1 (OR = 1.97 CI 95% 1.08–3.56; p-value = 0.022) and S2 group. In this last case, however, only a trend toward significance was detected (OR = 1.66 CI 95% 0.99–2.79; p-value = 0.054) (Table 2 and Figure 1B). For both SNPs, we did not find differences between age groups in comparison 1, indicating that carrying of the above alleles confers a disadvantageous or an advantageous effect on lifespan only in the last part of life. A different trajectory was observed for the *COMT* rs4680 variant (Figure 1C). Indeed, in all the comparisons, we found that the proportion of homozygous AA individuals was always significantly higher in the oldest than in the

younger subjects (OR = 1.67 CI 95% 1.10–2.54, *p*-value = 0.016 for comparison 1; OR = 2.43 CI 95% 1.58–3.73, *p*-value <0.001 for comparison 2; OR = 1.47CI 95% 1.10–2.15, *p*-value = 0.046 for comparison 3), consistent with a linear trend towards a positive effect of the rs4680-AA genotype on longevity. As for the rs2273697 variant in *ABCC2*, a significantly higher prevalence of carriers of the minor allele A in subjects belonging to the S3 group in comparison to the younger age group S2 (comparison 3) (OR = 1.459 CI 95% 1.038–2.051; *p*-value = 0.030) was observed (Figure 1D), thus indicating a beneficial impact of this allele for reaching longevity. No statistically significant evidence for genotype-by-sex interactions was observed.

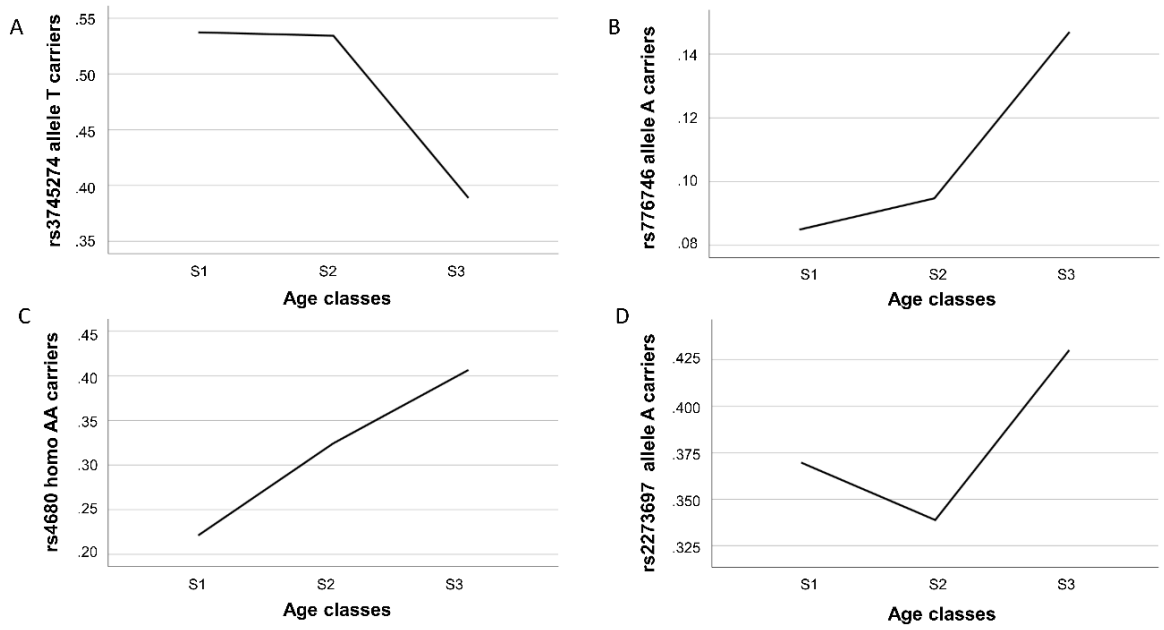

Figure 1. Gene frequencies across the three age classes S1 (20–64 years), S2 (65–89 years), and S3 (90–108 years) of: (**A**) T allele carriers of rs3745274 in *CYP2B6*; (**B**) A allele carriers of rs776746 in *CYP3A5*; (**C**) AA carriers of rs4680 in *COMT*; (**D**) A allele carriers of rs2273697 in *ABCC2*.

Next, to evaluate the overall effect of the multivariate model on the total phenotypic variance, we estimated Nagelkerke indexes for comparisons 1 and 3; we found that the variance explained by the combined genetic data was 0.7% in comparison 1 and 7.7% in comparison 3. Finally, we evaluated the combined effect of the variability of genes listed in Table 2 at different ages. As Table S2 shows, we detected an approximately similar effect size to that seen in univariate analysis, suggesting an independent effect of each SNP. In comparison 3, three out of four SNPs included in the genetic profile remained substantially associated with the phenotype and significantly associated with the age-group

membership (rs3745274-G/T, rs776746-G/A, rs4680-G/A), thus significantly discriminating very long lived (90+) from younger elderly (65–89 years old) subjects.

Since the weight of genetic factors increases starting from 65 years of age, we investigated the association of the above variants with biomarkers of age-associated changes in physical (HG and ADL) and cognitive (MMSE and GDS) abilities. A significant association was found between *COMT* rs4680 and ADL performance (*p*-value = 0.03) with subjects homozygous for the allele A showing significantly lower probability to be disabled than those carrying at least one G allele (60.7% of AA among the non-disabled vs. 39.3% of AA among the disabled). In addition, we found that the same variant significantly influenced the GDS performance in females. Subjects with the AA genotype were more represented among non-depressed than depressed individuals (78.9% vs. 51.6%; *p*-value = 0.017). No other significant association between these SNPs and geriatric parameters was observed.

Finally, by using 10-year follow-up survival data, we assessed whether the variants we found associated with the longevity phenotype also influenced the survival of the elderly cohort (age 65–89 years). Kaplan–Meier survival analysis showed a trend for a positive association with survival for carriers of the minor allele (A) of rs2273697 in *ABCC2* (*p*-value = 0.054; see Figure 2), a result consistent with the positive effect on longevity. However, the association did not hold significance when multivariate Cox proportional hazard regression analysis was performed (HR = 0.63, 95% CI: 0.35–1.16; *p*-value = 0.143).

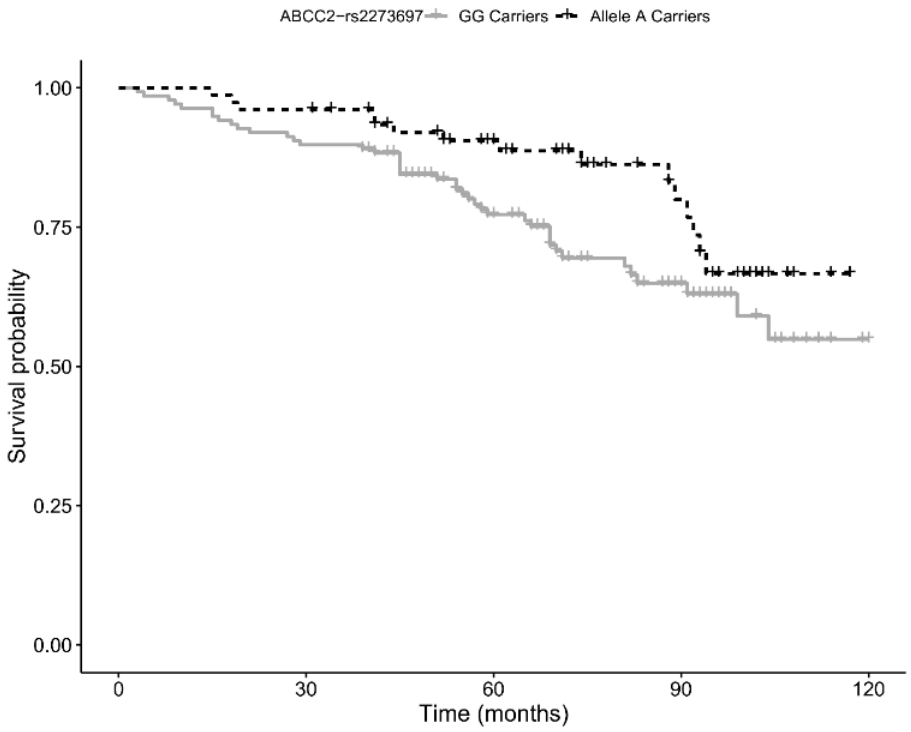

Figure 2. Kaplan–Meier survival functions relative to carriers of the minor allele A (black) vs. non-carriers (gray) of the *ABCC2* variant rs2273697. Time is expressed in months, where zero is considered the time of recruitment, and each individual is followed up for survival status until death.

4. Discussion

In this study, we show that genetic variants of genes related to xenobiotic metabolism, such as those of phases I-III, have an influence on the chance of reaching old and very old ages beyond 100 years. Among the 27 genetic variants analyzed, four (rs3745274, rs776746, rs4680, and rs2273697) have shown to exert significantly different and age-specific effects on longevity, with changes of gene frequencies following either linear or non-linear trajectories. In particular, except for rs4680, which showed a significant linear change across the age classes, we observed major frequency changes in the other SNPs in passing from 65–89 to 90–108 age-range. In fact, the genetic variability of these genes showed to account for 7.7% of the chance to survive beyond the age of 89 years. This figure is quite important if we take into account that genetics is believed to account for 25% of the individual chance to be long-lived.

Age-specific gene effects have already been reported in literature for genetic variants in other genes thought to shape the dynamic and the complex gene–environment interactions, which profoundly change

during human lifespan [21]. Genes encoding for xenobiotic metabolizing enzymes surely have these characteristics.

Completely opposite effects on longevity were observed for rs3745274-T (negative effect) and rs776746-A carriers (positive effect) located respectively in *CYP2B6* and *CYP3A5* genes that function in biotransformation reactions (phase I). Substrates for both isoenzymes include not only clinically used drugs but also a large number of environmental toxic and carcinogenic chemicals (pollutants, pesticides), as well as endogenous compounds such as steroid hormones and fatty acids [22,23].

The CYP2B6 protein makes up roughly 2–6% of total liver CYP content [24]. There is a remarkable inter-individual variability in its expression partly due to transcriptional regulation and genetic variations [22]. The rs3745274-G/T, herein found to affect the likelihood of becoming long-lived, is a missense variant (Gly516His) in exon 4. A study by Hofmann et al. [25] reported evidence that the T allele is responsible for aberrant splicing, resulting in a shorter variant lacking exon 4, 5, and 6 and decreased expression and enzymatic activity. Notably, relevant studies have established a link between this variant and a higher risk of multiple cancers [26–28]. Therefore, it is likely that individuals carrying the T allele, because of a lower detoxifying capability, have a higher susceptibility to cancer compared to non-carriers and thus a decreased probability of reaching advanced ages.

Emerging evidence also suggests that the rs776746-G/A variant in intron 3 of the *CYP3A5* gene may affect the individual's risk of cancer development. The presence of the G allele creates a cryptic splice site that determines a truncated non-functional protein. Therefore, subjects carrying the GG genotype are considered to be CYP3A5 non-expressors [29]. Very interestingly, the frequency of the rs776746-G allele varies markedly across ethnic groups, ranging from about 18% in Africans to 94% in Europeans (data from 1000 genome) and is significantly correlated with population distance from the equator [30], thus suggesting considerable interaction between genotype and environment. Notably, current literature supports a relationship between the G allele and cancer risk [31,32], suggesting that a protective effect of the expressor A-allele may allow individuals to better cope with dangerous compounds potentially promoting cancer development. Consistently, we found that elderly subjects carrying the rs776746-A have a higher likelihood of becoming long-lived than non-carrying ones. Interestingly, for both rs3745274 and rs776746, changes in gene frequencies start to occur in the middle-aged group (S2), i.e., the one characterized by a higher incidence of cancer and other age-related diseases.

Moreover, the polymorphism rs2273697-G/A appeared to modulate the probability of obtaining longevity by mainly acting in the 65–89 years age group. This is a missense variant (Val417Ile) in exon

10 of the *ABCC2* gene, encoding for the multidrug resistance-associated protein 2 (MRP2), a member of the ABC transporter superfamily. This phase III protein, which is expressed in hepatocytes, renal proximal tubular cells, and enterocytes, is involved in the removal of many toxic chemicals, nutraceuticals, drugs, and their conjugates, as well as endogenous compounds (e.g., bilirubin-glucuronides) [33]. A recent finding by Wei and colleagues provided direct evidence that the rs2273697-A allele increases the ATPase and the efflux activity of the MRP2 protein [34]. Our survival analysis showed that the presence of the A allele decreases the risk of death in subjects aged 65–89 years, and that carriers of this allele are more represented in the 90+ cohort. Based on the above evidence, it seems plausible that rs2273697-A promotes increased survival at old age through an enhanced MRP2 efflux capacity, which likely results in a better protection against cytotoxic effects of toxic compounds.

Finally, we observed a significant linear increase in frequency for the *COMT* rs4680-AA genotype across the different age groups, suggesting a positive effect on survival at both old and very old ages. The rs4680 polymorphism lies in exon 4 and determines either a valine (Val; G allele) or a methionine (Met; A allele) at amino acid 158. This SNP has been reported to significantly affect enzyme activity; for instance, the Val allele has a four-fold higher enzyme activity than the Met allele [35]. COMT is an important phase II enzyme, which, by methylation, inactivates biologically active catechols (i.e., catecholamines, catecholestrogens) and toxic catechol-based molecules [36]. In recent years, COMT has become intensively studied, largely due to its role in regulation of the dopamine level in the brain. In Caucasians, the Met allele is reported to be associated with better cognitive function [37], while Val carriers tend to have a greater likelihood of becoming depressed [38]. It is well known that depression as well as cognition impairment represent major risk factors for disability, often associated with worse health outcomes and increased risk of death, especially in later life [39]. All of the above is in line with our data showing a positive effect on longevity of the A (Met) allele, moreover, confirmed by the association of the same allele with a lower depressive status and a better physical performance in elderly subjects.

On the whole, the different trends in XME gene frequencies we observed in population age once again highlight the complexity in gene–longevity associations. In fact, we found two associated SNPs behaving as pro-longevity variants (rs776746-A and rs4680-AA), one as a killing variant (rs3745274-T), while the rs2273697-A allele showed a U-like frequency curve that was higher at younger ages, decreased in early old, and then increased in exceptionally old. Such a behavior is typical of a buffered

variant, in accordance with the buffering mechanism in aging hypothesis suggested by Bergman [40], which states that a deleterious variant can be neutralized by the protective effect of pro-longevity genes.

5. Conclusions

The present study—the first to our knowledge to investigate the association between SNPs in XME genes and human longevity—found that their variability conditions the chance to reach very old age by affecting survival in an age-specific way. This is a novel finding considering that the variability of XME genes has been extensively investigated in relation to drug metabolism and response to treatment. However, drugs are only a limited kind of substrate that XME proteins can metabolize; their detoxification function might rather modulate the dynamic and the complex biological response to the exposome, thus representing a potential determinant of longevity. We are aware that the study is not conclusive and deserves future investigations. Because the genetic variability of all the XME genes herein analyzed show population specificity, it could be very interesting to test the association with longevity in other ethnic groups. Moreover, a longer follow up time and the knowledge of specific causes of death could further support our conclusions, allowing us to better understand the specific pathological phenotype affected by the variants analyzed in this study. Nevertheless, the associations here reported may contribute to our understanding of the genetic determinants of human longevity, supporting future studies in the role of xenobiotic metabolism in quality of aging and extreme survival.

Supplementary Table S1. List of genes and SNPs considered in the present study.

XME	Gene	SNP	Functional Consequence	AA change
Phase I	CYP2A6	rs28399433 - A/C	2KB Upstream Variant	
	CYP2B6	rs3745274 - G/T	Missense Variant	Gln172His
	CYP2E1	rs2070673 - T/A	2KB Upstream Variant	
	CYP3A5	rs776746 - C/T	Intron /Splice Acceptor Variant	
	CYP2C19	rs4244285 - G/A	Synonymous variant	Pro227Pro
		rs12248560 - C/T	2KB Upstream Variant	
Phase II	COMT	rs165599 - A/G	3 Prime UTR Variant	
		rs4680 - G/A	Missense Variant	Val158Met
	GSTP1	rs1695 - A/G	Missense Variant	Ile105Val
	NAT2	rs1801280 - T/C	Missense Variant	Ile114Thr
		rs1799930 - G/A	Missense Variant	Arg197Gln
		rs1208 - A/G	Missense Variant	Arg268Lys
	UGT1A1	rs4124874 - T/G	Intron Variant	
	UGT2B7	rs7662029 - G/A	Intron Variant	
		rs7668258 - C/T	Intron Variant	
	UGT1A6	rs2070959 - A/G	Missense Variant	Thr181Ala
	UGT1A10	rs6759892 - T/G	Missense Variant	Ser158Ala
Phase III	ABCB1	rs2032582 - A/C	Missense Variant	Ser893Thr
		rs1128503 - G/A	Synonymous variant	Gly412Gly
	ABCC2	rs2273697 - G/A	Missense Variant	Val417Ile
		rs3740066 - C/T	Synonymous variant	Ile1324Ile
	ABCG2	rs2231142 - G/T	Missense Variant	Gln141Lys
	SLC15A2	rs2257212 - C/T	Missense Variant	Leu350Phe
		rs1143671 - C/T	Missense Variant	Pro409Ser
		rs1143672 - G/A	Missense Variant	Arg509Lys
	SLC22A2	rs316019 - C/A	Missense Variant	Ala270Ser
		rs316019 - C/A	Missense Variant	Ser270Ala
	SLCO1B1	rs4149056 - T/C	Missense Variant	Val174Ala
	SLCO1B3	rs4149117 - G/T	Missense Variant	Ser112Ala

		rs7311358 - A/G	Missense Variant	Met233Ile
Others*	DPYD	rs1801265 - A/G	Missense Variant	Cys29Arg
	ITGB3	rs5918 - T/C	Missense Variant	Leu59Pro
	PGTS1	rs5788 - C/A	Synonymous Variant	Gly213Gly
		rs10306114 - A/G	5 Prime UTR Variant	
	PTGS2	rs20417 - C/G	2KB Upstream Variant	

Underlined SNP were excluded from the analysis after quality control.

* These genes and relative polymorphisms were selected because deeply studied in relation to drug response, so likely affecting the risk or the clinical evolution of several diseases.

Supplementary Table S2. Multinomial logistic analysis for multivariate genetic associations.

		*Comparison 1 (Age class 2 *vs* Age class 1) 65-89 years vs <65 years		Comparison 2 (Age class 3 *vs* Age class 1) 90+ vs <65 years		Comparison 3 (Age class 3 *vs* Age class 2) 90+ vs 65-89 years	
	Gene	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
rs3745274-G/T	*CYP2B6*	1.09 (0.70-1.67)	0.702	0.62 (0.39-0.99)	0.045	0.54 (0.34-0.84)	0.006
rs776746-G/A	*CYP3A5*	1.03 (0.46-2.26)	0.946	1.86 (0.88-3.91)	0.101	1.93 (0.93-4.00)	0.075
rs4680-G/A	*COMT*	1.39 (0.84-2.32)	0.197	2.56 (1.56-4.21)	<0.001	1.93 (1.20-3.10)	0.007
rs2273697-G/A	*ABCC2*	0.97 (0.62-1.53)	0.914	1.28 (0.80-2.03)	0.30	1.30 (0.83-2.05)	0.253

*In each comparison the youngest group was considered as the reference category.

CI = Confidence interval. For both the comparisons 1 and 2 (both using the youngest group as reference category), Odd ratios (ORs) were obtained directly from the equations included in the models; for the comparison 3 (90+ years vs <65 years), ORs were obtained by difference of equations included in the models.

References

1. Wild, C.P. Complementing the genome with an "exposome": The outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol. Biomark. Prev.* 2005, 14, 1847–1850.
2. Escher, B.I.; Hackermüller, J.; Polte, T.; Scholz, S.; Aigner, A.; Altenburger, R.; Böhme, A.; Bopp, S.K.; Brack, W.; Busch, W.; et al. From the exposome to mechanistic understanding of chemical-induced adverse effects. *Environ. Int.* 2017, *99*, 97–106.
3. Xu, C.; Li, C.Y.; Kong, A.N. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* 2005, *28*, 249–268.
4. Klotz, U. Pharmacokinetics and drug metabolism in the elderly. *Drug Metab. Rev.* 2009, *41*, 67–76.
5. Wauthier, V.; Verbeeck, R.K.; Calderon, P.B. The effect of ageing on cytochrome P450 enzymes: Consequences for drug biotransformation in the elderly. *Curr. Med. Chem.* 2007, *14*, 745–757.
6. Yun, K.U.; Oh, S.J.; Oh, J.M.; Kang, K.W.; Myung, C.S.; Song, G.Y.; Kim, B.H.; Kim, S.K. Age-related changes in hepatic expression and activity of cytochrome P450 in male rats. *Arch. Toxicol.* 2010, *84*, 939–946.
7. Vyskočilová, E.; Szotáková, B.; Skálová, L.; Bártíková, H.; Hlaváčová, J.; Boušová, I. Age-related changes in hepatic activity and expression of detoxification enzymes in male rats. *BioMed Res. Int.* 2013, *2013*, 408573.
8. Corsonello, A.; Pedone, C.; Incalzi, R.A. Age-related pharmacokinetic and pharmacodynamic changes and related risk of adverse drug reactions. *Curr. Med. Chem.* 2010, *17*, 571–584.
9. McElwee, J.J.; Schuster, E.; Blanc, E.; Thomas, J.H.; Gems, D. Shared transcriptional signature in *Caenorhabditis elegans* Dauer larvae and long-lived *daf-2* mutants implicates detoxification system in longevity assurance. *J. Biol. Chem.* 2004, *279*, 44533–44543.
10. Gems, D.; McElwee, J.J. Broad spectrum detoxification: The major longevity assurance process regulated by insulin/IGF-1 signaling? *Mech. Ageing Dev.* 2005, *126*, 381–387.
11. Amador-Noguez, D.; Dean, A.; Huang, W.; Setchell, K.; Moore, D.; Darlington, G. Alterations in xenobiotic metabolism in the long-lived little mice. *Aging Cell* 2007, *6*, 453–470.
12. Hashibe, M.; Brennan, P.; Strange, R.C.; Bhisey, R.; Cascorbi, I.; Lazarus, P.; Ophuis, M.B.; Benhamou, S.; Foulkes, W.D.; Katoh, T.; et al. Meta- and pooled analyses of *GSTM1*, *GSTT1*, *GSTP1*, and *CYP1A1* genotypes and risk of head and neck cancer. *Cancer Epidemiol. Biomark. Prev.* 2003, *12*, 1509–1517.
13. Lee, K.M.; Kang, D.; Clapper, M.L.; Ingelman-Sundberg, M.; Ono-Kihara, M.; Kiyohara, C.; Min, S.; Lan, Q.; Le Marchand, L.; Lin, P.; et al. *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms, smoking, and lung cancer risk in a pooled analysis among Asian populations. *Cancer Epidemiol. Biomark. Prev.* 2008, *17*, 1120–1126.
14. Izzotti, A.; Cartiglia, C.; Lewtas, J.; De, F.S. Increased DNA alterations in atherosclerotic lesions of individuals lacking the *GSTM1* genotype. *FASEB J.* 2001, *15*, 752–757.
15. Ketelslegers, H.B.; Godschalk, R.W.; Gottschalk, R.W.; Knaapen, A.M.; Koppen, G.; Schoeters, G.; Baeyens, W.F.; Nelen, V.; Geraedts, J.P.; van Delft, J.H.; et al. Prevalence of at-risk genotypes for

genotoxic effects decreases with age in a randomly selected population in Flanders: A cross sectional study. *Environ. Health* 2011, *10*, 85.
16. De Rango, F.; Montesanto, A.; Berardelli, M.; Mazzei, B.; Mari, V.; Lattanzio, F.; Corsonello, A.; Passarino, G. To grow old in southern Italy: A comprehensive description of the old and oldest old in Calabria. *Gerontology* 2011, *57*, 327–334.
17. Passarino, G.; Montesanto, A.; Dato, S.; Giordano, S.; Domma, F.; Mari, V.; Feraco, E.; De Benedictis, G. Sex and age specificity of susceptibility genes modulating survival at old age. *Hum. Hered.* 2006, *62*, 213–220.
18. Folstein, M.F.; Folstein, S.E.; McHugh, P.R. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J. Psychiatr. Res.* 1975, *12*, 189–198.
19. Katz, S.; Ford, A.B.; Moskowitz, R.W.; Jackson, B.A.; Jaffe, M.W. Studies of illness in the aged. The index of ADL: A standardized measure of biological and psychosocial function. *JAMA* 1963, *185*, 914–919.
20. Lesher, E.L.; Berryhill, J.S. Validation of the Geriatric Depression Scale—Short Form among inpatients. *J. Clin. Psychol.* 1994, *50*, 256–260.
21. Giuliani, C.; Sazzini, M.; Pirazzini, C.; Bacalini, M.G.; Marasco, E.; Ruscone, G.A.G.; Fang, F.; Sarno, S.; Gentilini, D.; Di Blasio, A.M.; et al. Impact of demography and population dynamics on the genetic architecture of human longevity. *Aging (Albany NY)* 2018, *10*, 1947–1963.
22. Turpeinen, M.; Zanger, U.M. Cytochrome P450 2B6: Function, genetics, and clinical relevance. *Drug Metabol. Drug Interact.* 2012, *27*, 185–197.
23. Lamba, J.; Hebert, J.M.; Schuetz, E.G.; Klein, T.E.; Altman, R.B. PharmGKB summary: Very important pharmacogene information for *CYP3A5*. *Pharmacogenet. Genom.* 2012, *22*, 555–558.
24. Zanger, U.M.; Klein, K. Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): Advances on polymorphisms, mechanisms, and clinical relevance. *Front. Genet.* 2013, *4*, 24.
25. Hofmann, M.H.; Blievernicht, J.K.; Klein, K.; Saussele, T.; Schaeffeler, E.; Schwab, M.; Zanger, U.M. Aberrant splicing caused by single nucleotide polymorphism c.516G>T [Q172H], a marker of *CYP2B6*6*, is responsible for decreased expression and activity of CYP2B6 in liver. *J. Pharmacol. Exp. Ther.* 2008, *325*, 284–292.
26. Yuan, Z.H.; Liu, Q.; Zhang, Y.; Liu, H.X.; Zhao, J.; Zhu, P. *CYP2B6* gene single nucleotide polymorphisms and leukemia susceptibility. *Ann. Hematol.* 2011, *90*, 293–299.
27. Justenhoven, C.; Pentimalli, D.; Rabstein, S.; Harth, V.; Lotz, A.; Pesch, B.; Brüning, T.; Dörk, T.; Schürmann, P.; Bogdanova, N.; et al. *CYP2B6*6* is associated with increased breast cancer risk. *Int. J. Cancer* 2014, *134*, 426–430.
28. Daraki, A.; Zachaki, S.; Koromila, T.; Diamantopoulou, P.; Pantelias, G.E.; Sambani, C.; Aleporou, V.; Kollia, P.; Manola, K.N. The $G^{516}T$ *CYP2B6* germline polymorphism affects the risk of acute myeloid leukemia and is associated with specific chromosomal abnormalities. *PLoS ONE* 2014, *9*, e88879.
29. Perera, M.A. The missing linkage: What pharmacogenetic associations are left to find in CYP3A? *Expert Opin. Drug Metab. Toxicol.* 2010, *6*, 17–28.
30. Thompson, E.E.; Kuttab-Boulos, H.; Witonsky, D.; Yang, L.; Roe, B.A.; Di Rienzo, A. *CYP3A* variation and the evolution of salt-sensitivity variants. *Am. J. Hum. Genet.* 2004, *75*, 1059–1069.

31. Wang, B.S.; Liu, Z.; Xu, W.X.; Sun, S.L. *CYP3A5*3* polymorphism and cancer risk: A meta-analysis and meta-regression. *Tumor Biol.* 2013, *34*, 2357–2366.
32. Liang, Y.; Han, W.; Yan, H.; Mao, Q. Association of *CYP3A5*3* polymorphisms and prostate cancer risk: A meta-analysis. *J. Cancer Res. Ther.* 2018, *14*, S463–S467.
33. Jemnitz, K.; Heredi-Szabo, K.; Janossy, J.; Ioja, E.; Vereczkey, L.; Krajcsi, P. ABCC2/Abcc2: A multispecific transporter with dominant excretory functions. *Drug Metab. Rev.* 2010, *42*, 402–436.
34. Wei, D.; Zhang, H.; Peng, R.; Huang, C.; Bai, R. ABCC2 (1249G > A) polymorphism implicates altered transport activity for sorafenib. *Xenobiotica* 2017, *47*, 1008–1014.
35. Chen, J.; Lipska, B.K.; Halim, N.; Ma, Q.D.; Matsumoto, M.; Melhem, S.; Kolachana, B.S.; Hyde, T.M.; Herman, M.M.; Apud, J.; et al. Functional analysis of genetic variation in catechol-O-methyltransferase (COMT): Effects on mRNA, protein, and enzyme activity in postmortem human brain. *Am. J. Hum. Genet*. 2004, *75*, 807–821.
36. Männistö, P.T.; Kaakkola, S. Catechol-O-methyltransferase (COMT): Biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. *Pharmacol. Rev.* 1999, *51*, 593–628.
37. Aguilera, M.; Barrantes-Vidal, N.; Arias, B.; Moya, J.; Villa, H.; Ibanez, M.I.; Ruiperez, M.A.; Ortet, G.; Fananas, L. Putative role of the *COMT* gene polymorphism (Val158Met) on verbal working memory functioning in a healthy population. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 2008, *147B*, 898–902.
38. Wang, M.; Ma, Y.; Yuan, W.; Su, K.; Li, M.D. Meta-analysis of the *COMT* Val158Met polymorphism in major depressive disorder: effect of ethnicity. *J. Neuroimmune Pharmacol.* 2016, *11*, 434–445.
39. Mehta, K.M.; Yaffe, K.; Langa, K.M.; Sands, L.; Whooley, M.A.; Covinsky, K.E. Additive effects of cognitive function and depressive symptoms on mortality in elderly community-living adults. *J. Gerontol. A Biol. Sci. Med. Sci.* 2003, *58*, 461–467.
40. Bergman, A.; Atzmon, G.; Ye, K.; MacCarthy, T.; Barzilai, N.; Buffering mechanisms in aging: A systems approach toward uncovering the genetic component of aging. *PLoS Comput. Biol.* 2007, *3*, e170.

CHAPTER 3

Inositol Polyphosphate Multikinase (*IPMK*), a Gene Coding for a Potential Moonlighting Protein, Contributes to Human Female Longevity

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1. Introduction

In the last few decades, research on aging has seen progressive growth due to the social and medical burden correlated to the increase of the elderly population in developed countries. These efforts point towards a better understanding of the connections between aging, health, and longevity as they may provide useful insights for strategies to improve the wellbeing of the elderly. The results obtained in different research areas underscored the dynamic complexity of such connections [1]. These studies often identify genes involved in the regulation of the aging process that are also susceptibility loci of one or multiple age-related diseases. For instance, many genetic variants associated with increased disease risk are present with high frequency among the oldest individuals: this means that a disease "risk allele" can also be a pro-longevity variant [2,3]. In some cases, the same variant exhibits opposite effects on the development of different diseases, with potential differential impact on longevity [4,5]. Finally, genetic risk factors may change their impact on mortality risk during the life course, i.e., from detrimental in middle life to beneficial at advanced ages, very often in a gender-specific way [6,7]. This makes the identification of genes that robustly associate with longevity very challenging; in fact, despite the high number of studies aimed at highlighting the genetic contributors to long-life, only *APOE* and *FOXO3A* were consistently replicated in different populations [8].

Among the mechanisms that may account for this complexity are interactions between different genes (epistasis) or SNP–SNP interactions at gene level, gene–environment (internal and external) interactions and pleiotropic (including trade-off-like) effects of genes on different phenotypes. Alongside this, potential contributing factors could be genes coding for proteins with different, relevant and often unrelated functions, since they may integrate various cellular activities in space and time. This special category of multifunctional proteins defined as moonlighting proteins [9], does not include protein isoforms resulting from different RNA splice variants, gene fusions or proteins with pleiotropic effects [10]. A protein with potential moonlighting capability is the inositol polyphosphate multikinase (IPMK). This protein was initially discovered in budding yeast and named Arg82 for its ability to regulate arginine metabolism [11,12]. Mammalian IPMK has well-established roles in inositol phosphate metabolism as it converts inositol (1,4,5)-trisphosphate (IP_3) to IP_4 and IP_4 to IP_5 [13,14]. In addition to its kinase activity, IPMK can function as a nuclear phosphoinositide kinase (PI3-kinase), which produces PIP_3 from PIP_2 [15]. Through its PI3-kinase activity, IPMK activates Akt/PKB and its downstream signaling pathways [16]. In addition, it regulates several protein targets non-catalytically via protein–protein interactions, including cytosolic signaling factors such as the mammalian target of

rapamycin complex 1 (mTORC1) [17] and the energy-sensing protein kinase AMPK [18]. Recently, Kim et al. [19] revealed that IPMK acts as an important regulator of Toll-like receptor (TLR)-induced innate immunity through its interaction with the tumor necrosis factor receptor–associated factor 6 (TRAF6). At the nuclear level, IPMK acts as a transcriptional coactivator for *p53* [20] and for serum response factor (SRF) signaling [21]. Finally, IPMK functions in the export of mRNA from the nucleus to the cytoplasm [22–24].

To our knowledge, there are no studies investigating the genetic variability of *IPMK* gene in human complex phenotypes; just one SNP, rs12570088, near to *IPMK* locus, was found related to the susceptibility to Alzheimer's and Crohn diseases [25].

Given the compelling evidence demonstrating IPMK's multifunctional nature and thus its classification as a moonlighting protein, the present paper addresses the hypothesis that *IPMK* genetic variability affects human aging and longevity.

2. Materials and Methods

Population Sample

We analysed five hundred sixty-eight unrelated subjects (252 men and 316 women aged 64–105 years), born in Calabria (South Italy) and recruited in the entire region through several campaigns, as previously reported [26]. Their Calabrian ancestry was ascertained up to the third generation. At baseline, all subjects were free of the major age-related pathologies (e.g., cancer, type-2 diabetes and cardiovascular diseases). The study was approved by the Ethical Committee of the University of Calabria (on 9-9-2004). Written informed consent was obtained from the subjects in accordance with institutional requirements and the Declaration of Helsinki principles.

The analyses were performed considering two sex- and age-specific groups obtained according to the survival functions of the Italian population from 1890 onward [27]. The two "thresholds of longevity" used to define these age classes were 88 years for men and 91 years for women. These cut-offs correspond to the point after which a significant negative change in the slope of the survival curve of the Italian population occurs. In particular, in the present study males younger than 88 and females younger than 91 years will be defined as controls (N = 309, mean age 74 years), while males older than 88 and females older than 91 years will be defined as cases (N = 259, mean age 96.9 years).

SNP Selection and Genotyping

We performed genotyping of 14 SNPs mapping within and nearby the *IPMK* gene, prioritized by a tagging approach. Analysis was performed by SEQUENOM MassArray iPLEX technology according to the procedure previously reported [28]. Sequenom Typer 4.0 Software was used for the management and analysis of the collected data. About 10% of the samples were reanalyzed and the concordance rate of the genotypes was higher than 99%.

Quality Control

After genotyping, samples were subjected to a battery of quality control (QC) tests. At sample level, subjects with a proportion of missing genotypes higher than 10% were dropped from the analysis. At SNP level, SNPs were excluded if they had a significant deviation from Hardy–Weinberg equilibrium (HWE, $p < 0.05$) in the control sample, a Missing Frequency (MiF) higher than 10% and a Minor Allele Frequency (MAF) lower than 5%.

Functional Parameters

Disability

A modification of the Katz' Index of activities of daily living (ADL) was used to assess the management of four everyday activities (toileting, getting up from bed, rising from a chair, walking around) [29]. For the analysis, ADL scores were dichotomized as 1 if the subject was able to perform every activity and 0 otherwise.

Physical performance

Evaluation of Hand Grip strength (HG) was performed through a handheld dynamometer (SMEDLEY's dynamometer TTM, Tokyo, Japan) while the subject was sitting with the arm close to their body, by repeating the measure three times with the stronger hand. The maximum of these values was used in statistical analyses. When the test was not performed, it was indicated if it was because of physical disabilities or if the subject refused to participate.

Cognitive functioning

Screening of cognitive impairment was carried out by MMSE, a 30-point scale able to evaluate several different cognitive areas including memory, calculation, abstraction, judgment, visual–spatial ability and language [30]. MMSE scores range from 0 (lowest cognitive function) to 30 (highest cognitive function). MMSE scores were normalized for age and educational status, variables known to affect the result of the test.

Statistical Methods

For each SNP, allele and genotype frequencies were estimated by gene counting from the observed genotypes. Hardy–Weinberg equilibrium (HWE) was tested by Fisher's exact test. Pairwise measures of linkage disequilibrium (LD) between the analyzed loci was estimated by Haploview (https://www.broadinstitute.org/haploview/haploview). A logistic regression model was also used to evaluate the effect of genetic variability on the chance to reach very advanced age. Different genetic models (dominant, additive and recessive) were used to test association, using for each SNP the minor allele as reference. For each SNP the most likely genetic model was then estimated on the basis of minimum level of statistical significance (Wald test *p*-value).

As this study was exploratory, the *p*-values are reported without employing conservative statistical significance thresholding procedure (e.g., Bonferroni correction) as that could eliminate potentially important findings.

In order to evaluate if the detected effect of the polymorphisms on longevity may result in differential patterns of survival of the different relevant genotypes, we evaluated survival after 10 years from the baseline visit. Univariate survival analysis was carried out by the Kaplan–Meier method and survival curves compared by log-rank test. Subjects alive after the follow-up time were considered as censored, and this time was used as the censoring date in the survival analyses. In addition, hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated by using Cox proportional hazard models taking into account age as a confounder variable.

Pairwise measures of linkage disequilibrium (LD) between the analyzed loci were calculated by Plink 1.9 [31] and plotted with the Haploview version 4.2 [32]. The amount of LD was quantified by Lewontin's coefficient (D'). Haplotype-based association analysis within the generalized linear model (GLM) framework was used to model the effect of haplotypes on the probability to attain longevity, by the haplo.stats package of R. The haplo.score function of this package has been used to obtain the score statistics. Permutation-based *p*-values were used to evaluate the significance of the scores obtained (10,000 permutations).

Statistical analyses have been performed using SNPassoc and surv packages of R [33].

3. Results

Fourteen SNPs from about 76 kb genomic sequences spanning the *IPMK* gene were selected for examination by a tagging approach. The QC phase excluded three SNPs. In particular, two SNP were

excluded due to MiF data higher than 10% (rs1698392, rs2440854) and one because it did not satisfy the HWE (rs2275443). Figure 1A shows the eleven high quality SNPs that were tested for association with longevity with their corresponding gene position, while panel B depicts the degree of LD between pairs of SNPs.

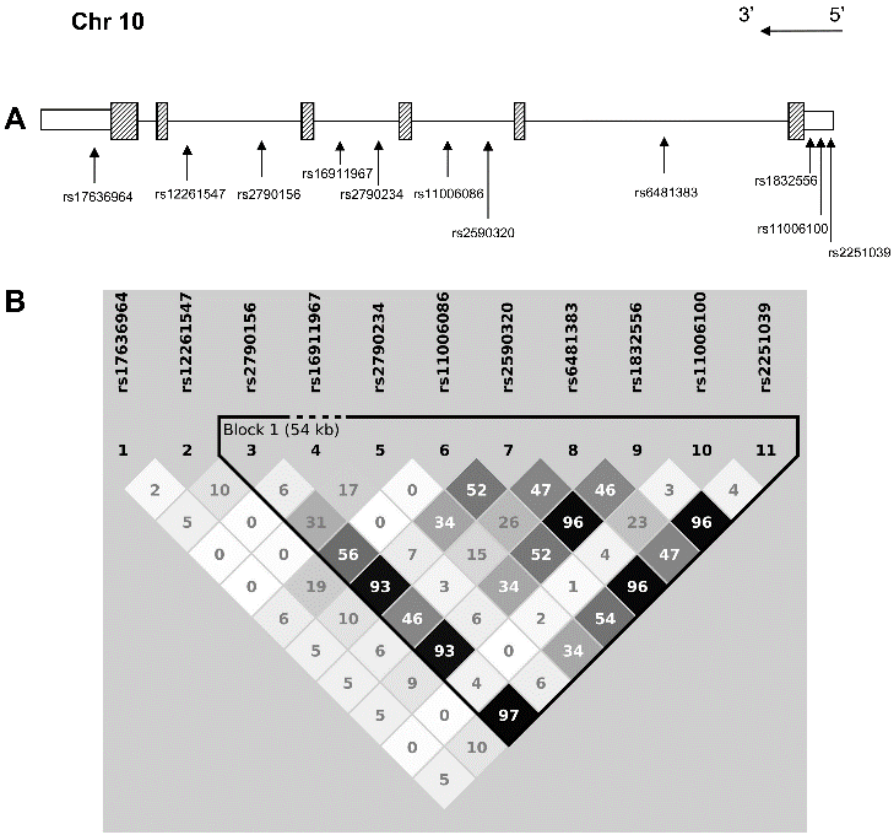

Figure 1. Schematic representation of (**A**) selected polymorphisms in the *IPMK* (Inositol Polyphosphate Multikinase) region; (**B**) linkage disequilibrium (r^2 coefficient) among the SNPs (Single Nuclear Polymorphisms).

3.1. Association with Longevity

Single SNP Analysis

The general characteristics of the analyzed sample are described in Table 1. Since a number of studies highlighted gender- and age-specific associations with survival at advanced age, in this work we analyzed the role of *IPMK* SNPs in the predisposition to become long-lived in gender subgroups.

Table 1. General characteristics and post-survey mortality in the analyzed sample.

	Elderly subjects	**Long-lived subjects**
N (age)	309 (74.06 ± 6.95)	259 (96.92 ± 3.72)
Females %	49.5%	63.0%
Height (cm)	160.6 (9.7)	151.4 (9.5)
BMI	26.9 (4.2)	23.21 (4.1)
HG strength [Kg (SD)]	21.89 (9.9)	13.04 (6.4)
ADL* (% Disabled)	17%	69%
MMSE	23.3 (5.4)	14.0 (6.8)

ADL, Activity Daily Living; HG, Hand Grip; BMI, Body Mass Index; MMSE, Mini Mental State Examination; for each parameter, mean value and standard deviation, in brackets, are shown. *Participants were defined as "not disabled" if independent in all items and "disabled" if dependent in at least one item.

From the results of the logistic regression analysis shown in Table 2, it can be seen that significant differences between the long-lived subjects and younger controls are present among females only. In particular, six out of eleven markers (in order: rs2790156-G/A, rs2790234-C/G, rs2590320-C/A, rs6481383-C/T, rs1832556-G/A, rs2251039-C/T) were significantly associated with the longevity phenotype under a dominant model of inheritance. For all the SNPs, the presence of the minor allele conferred decreased odds to reach advanced old age. rs2790234 showed the greatest impact [Odd Ratio (OR) 0.33; 95% Confidence Interval (CI) 0.16–0.67; P=0.00225], while association of similar magnitude was observed for the other five polymorphisms with ORs (95% CI) of 0.62, 0.572, 0.592, 0.592 and 0.612 (all p-values < 0.05) for rs2790156, rs2590320, rs6481383, rs1832556, rs2251039, respectively).

Table 2. Results of the logistic regression models for *IPMK* SNPs in the sample divided by sex.

a) Females			
SNP (Major/Minor Allele)	OR	95% CI	*p*-value
rs17636964 (G/C)	1,48	0,82-2,662	0,185
rs12261547 (G/C)	1,39	0,62-3,11	0,415
rs2790156 (G/A)	0,61	0,38-0,98	0,042
rs16911967 (G/C)	0,40	0,12-1,33	0,136
rs2790234 (C/G)	0,33	0,16-0,67	0,002
rs11006086 (T/C)	0,67	0,34-1,32	0,255
rs2590320 (C/A)	0,57	0,36-0,91	0,019
rs6481383 (C/T)	0,59	0,37-0,94	0,026
rs1832556 (G/A)	0,59	0,37-0,94	0,028
rs11006100 (T/A)	0,69	0,42-1,14	0,154
rs2251039 (C/T)	0,61	0,38-0,97	0,038
b) Males			
SNP	OR	95% CI	*p*-value
rs17636964 (G/C)	0,98	0,50-1,94	0,974
rs12261547 (G/C)	0,52	0,16-1,66	0,272
rs2790156 (G/A)	0,79	0,46-1,35	0,397
rs16911967 (G/C)	2,08	0,54-7,96	0,283
rs2790234 (C/G)	0,98	0,49-1,94	0,959
rs11006086 (T/C)	0,76	0,31-1,85	0,550
rs2590320 (C/A)	0,81	0,48-1,37	0,436
rs6481383 (C/T)	1,10	0,65-1,87	0,713
rs1832556 (G/A)	0,81	0,47-1,37	0,436
rs11006100 (T/A)	1,73	0,98-3,04	0,056
rs2251039 (C/T)	0,78	0,46-1,34	0,377

OR: Odd Ratio; CI: Confidence Interval

Haplotype-Based Analysis

To further explore the association of the entire region with longevity, we performed a haplotype analysis among the six SNPs associated with longevity. As shown in Figure 1B, all six SNPs lie in a large LD block, with rs2790156, rs2590320, rs1832556 and rs2251039 in strong LD and rs2790234 and rs6481383 in a weak linkage. Among all possible haplotypes, we found only four combinations: G-C-C-C-G-C, 61%; G-C-C-T-G-C, 16%; A-C-A-T-A-T, 14%; A-G-A-T-A-T, 7% (Table 3). In line with the single locus analysis, we found a negative association of the minor allele combination A-G-A-T-A-T with longevity in females (*p*-value = 0.002). On the contrary, a positive association was observed for the opposite combination G-C-C-C-G-C (*p*-value = 0.024). A deep analysis of the associated haplotypes showed that the strength of these associations was influenced by the allelic status at rs2790234 and rs6481383. Indeed, while A-**G**-A-T-A-T is significantly associated, the A-**C**-A-T-A-T is not; likewise, while G-C-C-**C**-G-C showed an effect on longevity, this was not true for G-C-C-**T**-G-C.

Table 3. Estimation of haplotype frequencies in the IPMK SNPs (in order: rs2790156, rs2790234, rs2590320, rs6481383, rs1832556, rs2251039) and association with longevity in the female sample.

Haplotype	Frequency	Score	*p*-value*
A-G-A-T-A-T	0.067	- 2.897	0.002
A-C-A-T-A-T	0.138	- 0.668	0.483
G-C-C-T-G-C	0.161	- 0.353	0.715
G-C-C-C-G-C	0.616	2.155	0.024

*****simulated *p*-value obtained by Monte Carlo replication up to 10,000 bootstraps.

Association with Survival

By using 10 years of follow-up survival data, we investigated if the single variants associated with female longevity also influenced the survival of the younger cohort. As shown in Figure 2, consistent with the detrimental effect on longevity, we found a trend toward significance for three out of six variants associated with longevity, rs2590320, rs1832556 and rs2251039, with HR values 1.72 (0.91–3.23), 1.75 (0.93–3.28), 1.75 (0.93–3.28) respectively ($p < 0.1$). we could not perform a haplotype-based survival analysis because a classification of carriers or non- carriers would reduce the size of the two classes too much.

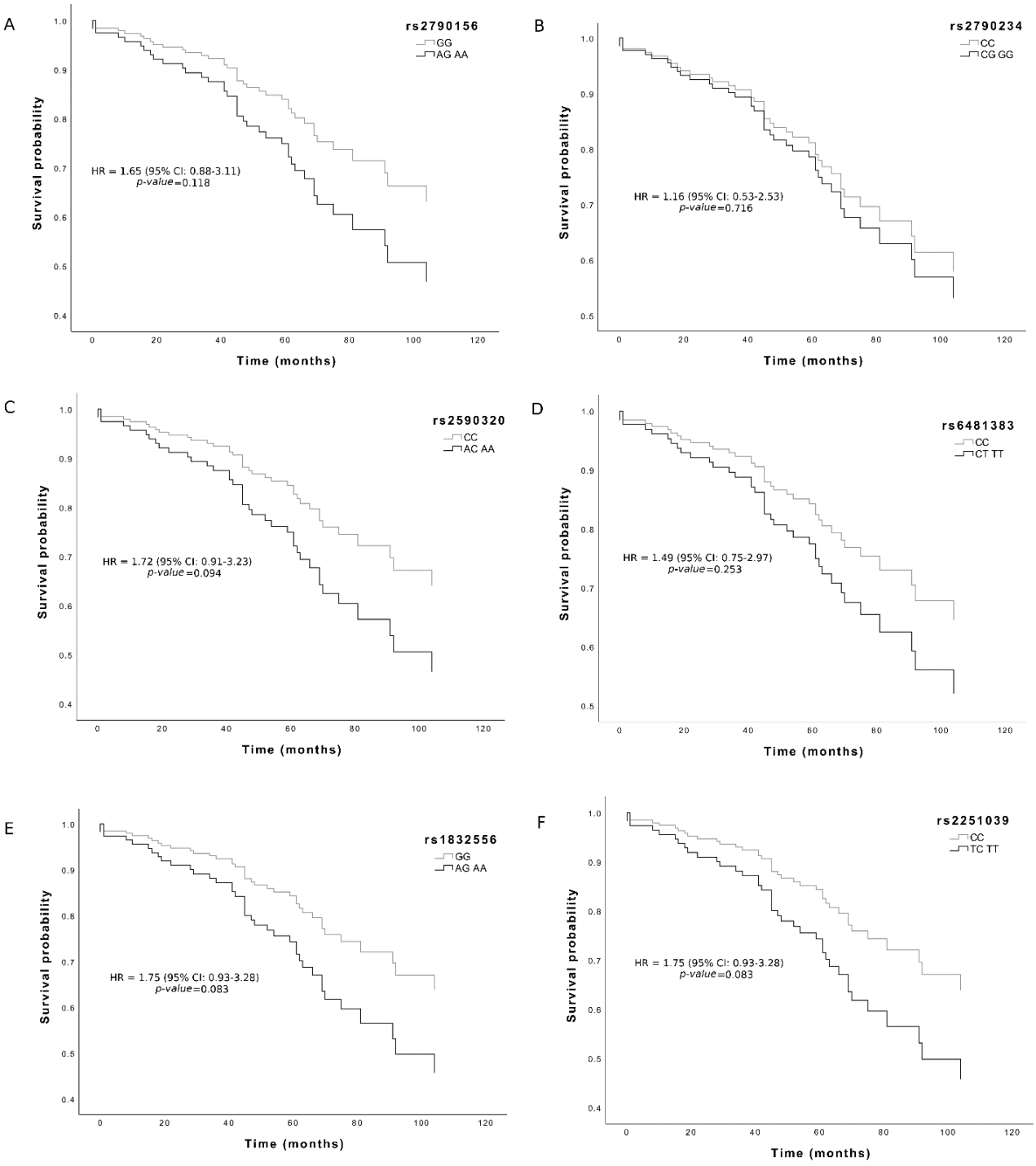

Figure 2. Survival functions of female carriers of minor allele (black) vs non-carriers (grey) of IPMK variants. (**A**) rs2790156; (**B**) rs2790234; (**C**) rs2590320; (**D**) rs6481383; (**E**) rs1832556; (**F**) rs2251039. Time is expressed in months, where 0 is considered the time of recruitment and each individual is followed up for survival status till death. The Cox regression was adjusted for age. HR (Hazard Ratio) value, confidence interval and *p*-value from Cox regression analysis are reported inside the figure.

Association with Functional Parameters

To investigate whether the variants in *IPMK* gene also concur to determine the age-related physiological decline, we analyzed the SNPs in relation to markers of physical (ADL and Hand Grip) and cognitive (MMSE) performance. No significant association was detected (data not shown). As a result, we conclude that *IPMK* has an effect on survival and longevity independently of the tested variables.

4. Discussion

Our study was designed to test the hypothesis that genetic variability at the *IPMK* locus contributes to survival to very old age. We provided evidence that polymorphisms in this gene significantly affect the females' chance of survival to old age, a result that implicates IPMK, a multifunctional protein with potential moonlighting functions, as a significant contributor to gender differences in longevity.

The gender difference in life expectancy and mortality, including survival to extreme age, as well as prevalence and incidence of the most important age-related diseases, is supported by a huge amount of clinical and demographic data [1, 34]. In almost all modern populations, females live longer than males and this has been attributed to a particular combination of genetic factors, environmental factors (nutrition and stress), sex hormones and immunity, along with socio-economic and cultural factors [33, 34]. Gender-specific longevity alleles were identified for a long time [37, 38] and recently confirmed by genome-wide association studies (GWAs) [39, 40]. These studies also indicated that different pathways contribute to longevity in men and women; for instance, paths involved in inflammation and immunity emerged as male-specific, while those involved in PGC-1α (PPARγ coactivator-1α) function and tryptophan metabolism emerged as female specific [40]. It is intriguing that many members of these pathways are known to perform diverse unrelated functions, behaving as moonlighting proteins [41, 42]. As previously discussed, proteins with moonlighting properties may, in part, explain the complex role of genetic factors in determining the longevity phenotype, including gender-linked effects. In this sense, IPMK is surely a possible player because of multifunctional protein feature. IPMK catalyzes key steps leading to the synthesis of inositol pyrophosphates [43]. These molecules, that as the name indicates contain one or more pyrophosphate moiety, control several aspects of cell physiology essential for cell survival. Inositol pyrophosphate regulate telomere length [44], vesicular trafficking [45], DNA recombination [46], ROS signaling [47] and energetic metabolism [48], likely by controlling cellular phosphate homeostasis [49, 50]. In fact, these molecules regulate the pathophysiology of metabolic disorders such diabetes and obesity [51, 52]. The numerous and distinct connections between metabolism and aging that research is highlighting [53, 54] suggest that inositol pyrophosphate might be

relevant to the aging process. Remarkably, the knockout of inositol hexakisphosphate kinase 3 (IP6K3), an essential enzyme for inositol pyrophosphates synthesis, results in altered metabolism and extends mice lifespan [55]. Furthermore, we associated two SNPs in the 5'-flanking promoter region of the *IP6K3* gene with the susceptibility to late onset Alzheimer's disease (LOAD) [28]. Therefore, the catalytic activity of IPMK, indispensable for inositol pyrophosphate synthesis, could be ultimately important for controlling metabolism and lifespan. Independently from its enzymatic activity, through protein–protein interaction, IPMK acts as a signaling hub in regulating nutrient and energetic pathways, including mTOR and AMPK [17, 56]. The inhibition of mTORC1 pathway components extends lifespan and confers protection against an increasing list of age-related diseases, while on the contrary its over-induction leads to a higher risk of age-related diseases and decreased lifespan [57, 58] and references therein]. A role in the control of cell survival and death has been also highlighted. A small deletion that leads a truncated form of IMPK was found to reduce the activation of *p53* and increase the resistance to apoptosis of cancer cell lines [59]. Moreover, Davey et al found that IPMK plays an important role in necroptosis, a form of regulated cell death prompted by injury and infection [60]. Both apoptosis and necroptosis impact on a variety of processes governing cell physiology and homeostasis with implications in health and disease [61].

It is interesting to note that many of the processes in which IPMK participates influence aging in a gender specific manner. For instance, some authors have suggested that gender differences in lifespan are due to gender-specific susceptibility to oxidative stress [62] and yet gender-specific survival is associated with sex differences in telomere dynamics [63]. It has also been demonstrated that rapamycin, the inhibitor of mTOR signaling, extends lifespan in dose- and gender-specific ways [64] and that there are sex-differences in muscle AMPK activation [65].

On the whole, these studies corroborate our findings that six *IPMK* SNPs significantly affect female longevity. Haplotype analysis confirmed the single SNP analyses, identifying an advantageous effect in carriers of the haplotype G-C-C-C-G-C; conversely the A-G-A-T-A-T haplotype is a disadvantageous combination for longevity. Haplotypes analysis allowed us to establish that among the six SNPs, two SNPs, rs2790234-C/G and rs6481383-C/T, were more likely to have an effect on lifespan, the minor rs2790234-G allele conferring a longevity disadvantage and the major rs6481383-C allele having a beneficial effect on the trait. The absence of correlation with survival and parameters of quality of aging suggests that these SNPs play a major role on the probability to achieve longevity.

Since we have no demonstrable functional explanation for the association as both SNPs occur in large intronic regions, it is difficult to evaluate their significance at this time. Moreover, both SNPs lie in a large region of LD, which likely contain hundreds of polymorphisms with one or more others that may have a direct contribution. Thus, our findings could be relevant for future investigations.

This study has some limitations that merit consideration. First, the sample size is rather small, which limits its statistical power. The sample size might have influenced the significance of survival analysis, so only trends have been identified. However, this result may also depend on the follow-up time of 10 years, not sufficient to draw long-term conclusions on the effect of genetic variants with a minor effect on survival. Therefore, the sample size should be increased and further explorations in additional populations and other countries are needed before drawing further conclusions. Another possible drawback is the lack of a proper correction for multiple testing. However, it should be noted that this was a pilot study, the first to analyze the association of *IPMK* variability in human aging and longevity, so a Bonferroni correction would have eliminated potentially important findings if applied. Furthermore, because associated SNPs are intronic, experimental evidence in support of the hypothesis that the detrimental genotypes influence the function of the protein should be carried out.

5. Conclusions

Specific *IPMK* haplotypes affect lifespan in women. Although our studies do not allow definitive conclusions, we believe that our findings can provide a basis for future studies to better clarify the basic mechanisms linking *IPMK* to female longevity and potential targets for realizing gender-specific therapeutic interventions. Finally, we believe that proteins with moonlighting capabilities, such as IPMK, could represent one of the factors that makes it difficult to disentangle the genetic complexity of the longevity phenotype.

References

1. Ukraintseva, S.; Yashin, A.; Arbeev, K.; Kulminski, A.; Akushevich, I.; Wu, D.; Joshi, G.; Land, K.C.; Stallard, E. Puzzling role of genetic risk factors in human longevity: "Risk alleles" as pro-longevity variants. *Biogerontology*. 2016, *17*, 109–127.
2. Mooijaart, S.P.; van Heemst, D.; Noordam, R.; Rozing, M.P.; Wijsman, C.A.; de Craen, A.J.; Westendorp, R.G.; Beekman, M.; Slagboom, P.E. Polymorphisms associated with type 2 diabetes in familial longevity: The Leiden Longevity Study. *Aging.* 2011, *3*, 55–62.
3. Sebastiani, P.; Riva, A.; Montano, M.; Pham, P.; Torkamani, A.; Scherba, E.; Benson, G.; Milton, J.N.; Baldwin, C.T.; Andersen, S.; Schork, N.J.; Steinberg, M.H.; Perls, T.T. Whole genome sequences of a male and female supercentenarian, ages greater than 114 years. *Front. Genet.*. 2011, *2*, 90.
4. Lio, D.; Scola, L.; Crivello, A.; Colonna-Romano, G.; Candore, G.; Bonafè, M.; Cavallone, L.; Franceschi, C.; Caruso, C. Gender-specific association between -1082 IL-10 promoter polymorphism and longevity. *Genes Immun*. 2002, *3*, 30–33.
5. Kulminski, A.M.; Culminskaya, I.; Arbeev, K.G.; Ukraintseva, S.V.; Arbeeva, L.; Yashin, A.I. Trade-off in the effect of the APOE gene on the ages at onset of cardiovascular disease and cancer across ages, gender, and human generations. *Rejuvenation Res.* 2013, *16*, 28–34.
6. Petersen, L.K.; Christensen, K.; Kragstrup, J. Lipid-lowering treatment to the end? A review of observational studies and RCTs on cholesterol and mortality in 80+-year olds. *Age Ageing* 2010, *39*, 674–80.
7. van Vliet, P.; Oleksik, A.M; van Heemst, D.; de Craen, A.J.M.; Westendorp, R.G.J. Dynamics of traditional metabolic risk factors associate with specific causes of death in old age. *J. Gerontol. Ser. A* 2010, *65*, 488–94.
8. Zeng, Y.; Nie, C.; Min, J.; Liu, X.; Li, M.; Chen, H.; Xu, H.; Wang, M.; Ni, T.; Li, Y.; et al. Novel loci and pathways significantly associated with longevity. *Sci. Rep.* 2016, *6*, 21243.
9. Jeffery, C.J. Moonlighting proteins. *Trends Biochem. Sci.* 1999, *24*, 8–11.
10. Jeffery, C.J. Molecular mechanisms for multitasking: recent crystal structures of moonlighting proteins. *Curr. Opin. Struct. Biol.* 2004, *14*, 663–668.
11. Bosch, D.; Saiardi, A. Arginine transcriptional response does not require inositol phosphate synthesis. *J. Biol. Chem*. 2012, *287*, 38347–38355.
12. Hatch, A.J.; Odom, A.R.; York, J.D. Inositol phosphate multikinase dependent transcriptional control. *Adv. Biol. Regul.* 2017, *64*, 9–19.
13. Saiardi, A.; Nagata, E.; Luo, H.R.; Sawa, A.; Luo, X.; Snowman, A.M.; Snyder, S.H. Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. *Proc. Natl. Acad. Sci. USA*. 2001, *98*, 2306–2311.
14. Seacrist, C.D.; Blind, R.D. Crystallographic and kinetic analyses of human IPMK reveal disordered domains modulate ATP binding and kinase activity. *Sci. Rep.* 2018, *8*, 16672.
15. Resnick, A.C.; Snowman, A.M.; Kang, B.N.; Hurt, K.J.; Snyder, S.H.; Saiardi, A. Inositol polyphosphate multikinase is a nuclear PI3-kinase with transcriptional regulatory activity. *Proc. Natl. Acad. Sci. USA.* 2005, 102 12783-8.

16. Maag, D.; Maxwell, M.J.; Hardesty, D.A.; Boucher, K.L.; Choudhari, N.; Hanno, A.G.; Ma, J.F.; Snowman, A.S.; Pietropaoli, J.W.; Xu, R.; Storm, P.B.; Saiardi, A.; Snyder, S.H.; Resnick, A.C. Inositol polyphosphate multikinase is a physiologic PI3-kinase that activates Akt/PKB. *Proc. Natl. Acad. Sci. USA.* 2011, *108*, 1391–1396.
17. Kim, S.; Kim, S.F.; Maag, D.; Maxwell, M.J.; Resnick, A.C.; Juluri, K.R.; Chakraborty, A.; Koldobskiy, M.A.; Cha, S.H.; Barrow, R.; Snowman, A.M.; Snyder, S.H. Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. *Cell Metab.* 2011, *2*, 215–221.
18. Dailey, M.J.; Kim, S. Inositol polyphosphate multikinase: An emerging player for the central action of AMP-activated protein kinase. *Biochem. Biophys. Res. Commun.* 2012, *421*, 1–3.
19. Kim, E.; Beon, J.; Lee, S.; Park, S.J.; Ahn, H.; Kim, M.G.; Park, J.E.; Kim, W.; Yuk, J.M.; Kang, S.J.; Lee, S.H.; Jo, E.K.; Seong, R.H.; Kim, S. Inositol polyphosphate multikinase promotes Toll-like receptor-induced inflammation by stabilizing TRAF6. *Sci. Adv.* 2017, 3, e1602296.
20. Xu, R.; Sen, N.; Paul, B.D.; Snowman, A.M.; Rao, F.; Vandiver, M.S.; Xu, J.; Snyder, S.H. Inositol polyphosphate multikinase is a coactivator of p53-mediated transcription and cell death. *Sci. Signal*. 2013, 6, ra22.
21. Kim, E.; Tyagi, R.; Lee, J.Y.; Park, J.; Kim, Y.R.; Beon, J.; Chen, P.Y.; Cha, J.Y.; Snyder, S.H.; Kim, S. Inositol polyphosphate multikinase is a coactivator for serum response factor-dependent induction of immediate early genes. *Proc. Natl. Acad. Sci. USA.* 2013, *110*, 19938–19943.
22. Saiardi, A.; Caffrey, J.J.; Snyder, S.H.; Shears, S.B. Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in Saccharomyces cerevisiae. *FEBS Lett.* 2000, *468*, 28–32.
23. Carmody, S.R.; Wente, S.R. mRNA nuclear export at a glance. *J. Cell. Sci.* 2009, *122*, 1933–1937.
24. Kim, E.; Beon, J.; Lee, S.; Park, J.; Kim, S. IPMK: A versatile regulator of nuclear signaling events. *Adv. Biol. Regul*. 2016, *61*, 25–32.
25. Yokoyama, J.S.; Wang, Y.; Schork, A.J.; Thompson, W.K.; Karch, C.M.; Cruchaga, C.; McEvoy, L.K.; Witoelar, A.; Chen, C.H.; Holland, D. et al. Alzheimer's Disease Neuroimaging Initiative. Association Between Genetic Traits for Immune-Mediated Diseases and Alzheimer Disease. *JAMA Neurol*. 2016, *73*, 691–97.
26. De Rango, F.; Montesanto, A.; Berardelli, M.; Mazzei, B.; Mari, V.; Lattanzio, F.; Corsonello, A.; Passarino, G. To grow old in southern Italy: a comprehensive description of the old and oldest old in Calabria. *Gerontology*. 2011, *57*, 327–334.
27. Passarino, G.; Montesanto, A.; De Rango, F.; Garasto, S.; Berardelli, M.; Domma, F.; Mari, V.; Feraco, E.; Franceschi, C.; De Benedictis, G. A cluster analysis to define human aging phenotypes. *Biogerontology*. 2007, *8*, 283–290.
28. Crocco, P.; Saiardi, A.; Wilson, M.S.; Maletta, R.; Bruni, A.C.; Passarino, G.; Rose, G. Contribution of polymorphic variation of inositol hexakisphosphate kinase 3 (IP6K3) gene promoter to the susceptibility to late onset Alzheimer's disease. *Biochim. Biophys. Acta.* 2016, *1862*, 1766–1773.
29. Katz, S.; Ford, A.B.; Moskowitz, R.W.; Jackson, B.A.; Jaffe, M.W. Studies of illness in the aged. The index of Adl: A standardized measure of biological and psychosocial function. *JAMA.* 1963, *185*, 914–919.

30. Folstein, M.F.; Folstein, S.E.; McHugh, P.R. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res.* 1975, *12*, 189–198.
31. Plink 1.9 home. Available online: https://www.cog-genomics.org/plink2) (accessed on 8 February 2019).
32. Barrett, J.C.; Fry, B.; Maller, J.; Daly, M.J. Haploview: Analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005, *21*, 263–265.
33. The R Project for Statistical Computing. Available online: http://www.R-project.org/ (accessed on 8 February 2019).
34. Austad, S.N.; Fischer, K.E. Sex Differences in Lifespan. *Cell Metab.* 2016, *23*, 1022–1033.
35. Ostan, R.; Monti, D.; Gueresi, P.; Bussolotto, M.; Franceschi, C.; Baggio, G. Gender, aging and longevity in humans: An update of an intriguing/neglected scenario paving the way to a gender-specific medicine. *Clin. Sci. (Lond).* 2016, *130*, 1711–1725.
36. Dato, S.; Rose, G.; Crocco, P.; Monti, D.; Garagnani, P.; Franceschi, C.; Passarino, G. The genetics of human longevity: An intricacy of genes, environment, culture and microbiome. *Mech. Ageing Dev.* 2017, *165*, 147–155.
37. Garasto, S.; Rose, G.; Derango, F.; Berardelli, M.; Corsonello, A.; Feraco, E.; Mari, V.; Maletta, R.; Bruni, A.; Franceschi, C.; Carotenuto, L.; De Benedictis, G. The study of APOA1, APOC3 and APOA4 variability in healthy ageing people reveals another paradox in the oldest old subjects. *Ann. Hum. Genet.* 2003, *67*, 54–62.
38. Altomare, K.; Greco, V.; Bellizzi, D.; Berardelli, M.; Dato, S.; De Rango, F.; Garasto S.; Rose, G.; Feraco, E.; Mari, V. et al. The allele (A) (-110) in the promoter region of the HSP70-1 gene is unfavorable to longevity in women. *Biogerontology.* 2003, *4*, 215–220.
39. Deelen, J.; Beekman, M.; Uh, H.W.; Helmer, Q.; Kuningas, M.; Christiansen, L.; Kremer, D; van der Breggen, R.; Suchiman, H.E.; Lakenberg, N. et al. Genome-wide association study identifies a single major locus contributing to survival into old age; the APOE locus revisited. *Aging Cell.* 2011, *10*, 686–698.
40. Zeng, Y.; Nie, C.; Min, J.; Chen, H.; Liu, X.; Ye, R.; Chen, Z.; Bai, C.; Xie, E.; Yin, Z. et al. Sex Differences in Genetic Associations With Longevity. *JAMA Netw. Open.* 2018, 1, e181670.
41. Jeffery, C.J. Moonlighting proteins--an update. *Mol Biosyst.* 2009, *5*, 345–350.
42. Houtkooper, R.H.; Williams, R.W.; Auwerx, J. Metabolic networks of longevity. *Cell*. 2010, *142*, 9–14.
43. Resnick, A.C.; Saiardi, A. Inositol polyphosphate multikinase: Metabolic architect of nuclear inositides. *Front. Biosci.* 2008; *13*; 856–866.
44. Saiardi, A.; Resnick, A.C.; Snowman, A.M.; Wendland, B.; Snyder, S.H. Inositol pyrophosphates regulate cell death and telomere length through phosphoinositide 3-kinase-related protein kinases. *Proc. Natl. Acad. Sci. USA.* 2005; *102*; 1911–1914.
45. Saiardi, A.; Sciambi, C.; McCaffery, J.M.; Wendland, B.; Snyder, S.H. Inositol pyrophosphates regulate endocytic trafficking. *Proc. Natl. Acad. Sci. USA.* 2002; *99*; 14206–14211.
46. Jadav, R.S.; Chanduri, M.V.; Sengupta, S.; Bhandari, R. Inositol pyrophosphate synthesis by inositol hexakisphosphate kinase 1 is required for homologous recombination repair. *J. Biol Chem*. 2013; *288*; 3312–3321.

47. Onnebo, S.M.; Saiardi, A. Inositol pyrophosphates modulate hydrogen peroxide signalling. *Biochem. J.* 2009; *423*; 109–118.
48. Szijgyarto, Z.; Garedew, A.; Azevedo, C.; Saiardi, A. Influence of inositol pyrophosphates on cellular energy dynamics. *Science.* 2011; *334*; 802–805.
49. Wild, R.; Gerasimaite, R.; Jung, J.Y.; Truffault, V.; Pavlovic, I.; Schmidt, A.; Saiardi, A.; Jessen, H.J.; Poirier, Y.; Hothorn, M.; Mayer, A. Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. *Science.* 2016; *352*; 986–990.
50. Azevedo, C.; Saiardi, A. Eukaryotic Phosphate Homeostasis: The Inositol Pyrophosphate Perspective. Trends *Biochem. Sci.* 2017; *42*; 219–231.
51. Illies, C.; Gromada, J.; Fiume, R.; Leibiger, B.; Yu, J.; Juhl, K.; Yang, S.N.; Barma, D.K.; Falck, J.R.; Saiardi, A. et al. Requirement of inositol pyrophosphates for full exocytotic capacity in pancreatic beta cells. *Science.* 2007; *318*; 1299–1302.
52. Zhu, Q.; Ghoshal, S.; Rodrigues, A.; Gao, S.; Asterian, A.; Kamenecka, T.M.; Barrow, J.C.; Chakraborty, A. Adipocyte-specific deletion of Ip6k1 reduces diet-induced obesity by enhancing AMPK-mediated thermogenesis. *J. Clin. Invest.* 2016; *126*; 4273–4288.
53. Finkel, T. The metabolic regulation of aging. *Nat. Med.* 2015; *21*; 1416–1423.
54. Dato, S.; Bellizzi, D.; Rose, G.; Passarino, G. The impact of nutrients on the aging rate: A complex interaction of demographic; environmental and genetic factors. *Mech. Ageing Dev.* 2016; 154:49-61,.
55. Moritoh, Y.; Oka, M.; Yasuhara, Y.; Hozumi, H.; Iwachidow, K.; Fuse, H.; Tozawa, R. Inositol Hexakisphosphate Kinase 3 Regulates Metabolism and Lifespan in Mice. *Sci. Rep.* 2016; *6*; 32072.
56. Bang, S.; Chen, Y.; Ahima, R.S.; Kim, S.F. Convergence of IPMK and LKB1-AMPK signaling pathways on metformin action. *Mol. Endocrinol.* 2014; *28*; 1186–1193.
57. Stanfel, M.N.; Shamieh, L.S.; Kaeberlein, M.; Kennedy, B.K. The TOR pathway comes of age. *Biochim Biophys Acta.* 2009; *1790*; 1067–1074.
58. Dato, S.; Hoxha, E.; Crocco, P.; Iannone, F.; Passarino, G.; Rose, G. Amino acids and amino acid sensing: Implication for aging and diseases. *Biogerontology.* 2019; *20*; 17–31.
59. Sei, Y.; Zhao, X.; Forbes, J.; Szymczak, S.; Li, Q.; Trivedi, A.; Voellinger, M.; Joy, G.; Feng, J.; Whatley, M. et al. A Hereditary Form of Small Intestinal Carcinoid Associated with a Germline Mutation in Inositol Polyphosphate Multikinase. *Gastroenterology*. 2015; *149*; 67–78.
60. Dovey, C.M.; Diep, J.; Clarke, B.P.; Hale, A.T.; McNamara, D.E.; Guo, H.; Brown, N.W. Jr; Cao, J.Y.; Grace, C.R.; Gough, P.J. et al. MLKL Requires the Inositol Phosphate Code to Execute Necroptosis. *Mol. Cell.* 2018; *70*; 936-948.e7.
61. Tower, J. Programmed cell death in aging. *Ageing Res. Rev*. 2015; *23*; 90–100.
62. Kander, M.C.; Cui, Y.; Liu, Z. Gender difference in oxidative stress: A new look at the mechanisms for cardiovascular diseases. *J. Cell Mol. Med.* 2017; *21*; 1024–1032.
63. Gardner, M.; Bann, D.; Wiley, L.; Cooper, R.; Hardy, R.; Nitsch, D.; Martin-Ruiz, C.; Shiels, P.; Sayer, A.A.; Barbieri, M. et al. Halcyon study team. Gender and telomere length: Systematic review and meta-analysis. *Exp. Gerontol.* 2014; *51*; 15–27.

64. Miller, R.A.; Harrison, D.E.; Astle, C.M.; Fernandez, E.; Flurkey, K.; Han, M.; Javors, M.A.; Li, X.; Nadon, N.L.; Nelson, J.F. et al: Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. *Aging Cell* 2014; *13*; 468–477.
65. Roepstorff, C.; Thiele, M.; Hillig, T.; Pilegaard, H.; Richter, E.A.; Wojtaszewski, J.F.; Kiens, B. Higher skeletal muscle alpha2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J. Physiol.* 2006; *574*; 125–138.

CHAPTER 4

IP6K3 and *IPMK* variations in LOAD and longevity: evidence for a multifaceted signaling network at the crossroad between neurodegeneration and survival

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1. Introduction

The heterogeneity in age-related functional decline is a highly debated topic (Field et al, 2018; Lowsky et al, 2014). The understanding of genetic and non-genetic factors that affect such heterogeneity is fundamental for the development of strategies to attenuate age related decline and prolong a healthy life. The study of centenarians, exceptionally long-lived individuals that in most cases experienced a delayed aging, has arisen growing interest for its potential to reveal information on the combination of genes and lifestyle factors that can prevent or postpone age-related diseases. Numerous researches in this field demonstrated that longevity is a highly plastic and dynamic trait being the result of a lifelong remodeling process which depends on a complex genetic architecture, influenced by extensive genotype-by-genotype and genotype-by-environment interactions (Dato et al, 2017). Undeniably, centenarians represent an extreme phenotype of good health and they could be considered as super-controls to compare the distribution of risk alleles with respect to patients with age related diseases, such as type two diabetes (T2D), assuming that such alleles should have the highest frequency among patients, an intermediate frequency among healthy subject and the lowest among centenarians (Garagnani et al, 2013). On the other hand, the systematic analysis of the 'gerontome', the collection of over 2000 genes shown to modulate aging in model organisms and human, suggested complex relationships between aging-related genes and age-related diseases. (Fernandes et al, 2016). For instance, many genetic variants associated with increased risks of diseases are found in genomes of long-lived people, suggesting they might be risk factors or protective factors according to other (genomic or environmental) concurrent factors (Beekman et al, 2010; Mooijaart et al, 2011; Raule et al, 2014; Sebastiani et al, 2013; Freudenberg-Hua et al, 2014). Similarly, some genetic alleles show tradeoff-like effect on mortality risk during life course (i.e. risk factors at adult age and pro longevity at advanced ages), or the same variant shows opposite effects on different age-related diseases, differently affecting the individual mortality risk (Ukraintseva et al, 2016).

To understand this genetic complexity, we need to consider that the biological systems function as networks of biomolecules; this implies that the individual effect of a gene can be negative or positive, or even neutral, depending on the interactions occurring with components of the different networks, which may change along life progression. Primarily relevant to this scenario are signaling molecules or proteins with multiple functions which may act as either key signaling hubs or "switchers" connecting different pathways, influencing several cellular functions essential for survival. Past evidence suggests energy production and storage, may be crucial in this context. Indeed, the variability of mitochondrial

DNA, affecting subunits of the oxidative phosphorylation chain, has been found to be correlated to longevity as well as diseases associated to tissue (especially neuronal) degeneration. In several studies the same allelic variation in mtDNA was associated to both longevity and to degenerative diseases (Raule et al. 2014 and references therein). Similarly, mitochondrial uncoupling proteins 4 (*UCP4*) variation (rs9472817), affecting the management of energy, has been found to affect both longevity and neurodegenerative disease (Rose et al, 2011; Montesanto et al, 2016; Montesanto et al, 2018).

Inositol polyphosphates and specifically the pyrophosphate containing species are emerging as molecules playing key role in the management of energy homeostasis and with fundamental role in regulating multiple cellular process (Tsui and York, 2010; Livermore et al, 2016). The energy-rich inositol pyrophosphates IP7 and IP8, generated by sequential phosphorylation of the calcium releasing factor IP3 or from the glycolytic intermediate glucose-6P (Desfougères et al, 2019), display pleiotropic effects acting as potential 'molecular switch' in the regulation of a wide spectrum of central processes such as phosphate homoeostasis and energetic metabolism (Azevedo and Saiardi, 2017; Thota and Bhandari, 2015; Wilson et al, 2013; Wundenberg and Mayr, 2012). Hence, inositol pyrophosphates may be crucial at the "crossroad" of age-related disease and longevity networks. In mammalian cells, the pathway of inositol pyrophosphates synthesis involves multiple enzymatic steps carried by five different type of kinases; the ITPK1 type of multi kinase, the inositol polyphosphate multi kinase (IPMK), the inositol pentakisphosphate kinase IPPK, three different isoforms of inositol hexakisphosphate kinase (IP6K1–3) and two different isoforms of the diphosphoinositol pentakisphosphate kinase (PPIP5K1-2).

Noteworthy is the capacity of many of these kinases and especially of both IPMK and IP6K3 to, independently of their ability to generate inositol pyrophosphates, act on several protein targets non-catalytically via protein–protein interactions, influencing multiple different biological processes (Rojas et al, 2019; Kim et al, 2017; Fu et al, 2015). The multifunctionality of these proteins, is suggestive of a fine tuning of the inositol phosphates signaling network during life progression. It is possible to hypothesize that the complexity and adaptability of the inositol phosphate signaling pathways contributed to the heterogeneity in the age-related functional decline with variability in the *IPMK* and *IP6K3* genes that could be leading either to neurodegeneration or to a long life.

Such hypothesis is supported by different evidences; a rare variant (rs12570088) near to *IPMK* locus is related to the susceptibility to Alzheimer's disease (Yokoyama et al 2016); IPMK acts as a regulator of fear extinction and synaptic plasticity (Park et al, 2019); furthermore, there are evidence that IP6K3 is linked to lifespan in mice (Moritoh et al, 2016).

We recently demonstrate that the genetic variability of *IPMK* affect human longevity, by reporting a six-SNPs haplotype that significantly influences female longevity (De Rango et al, 2019). Additionally, a study by Crocco et al (2016) showed that the variability of *IP6K3*, which is highly expressed in the brain, is associated with increased risk of late onset Alzheimer's disease (LOAD).

Considering that longevity and neurodegeneration share some relevant pathways, in the same sample groups analyzed in Crocco et al (2016) and De Rango et al, (2019) here we investigated whether: a) the genetic variability of *IP6K3*, previously associated with LOAD can affect the ability to live longer, and b) the genetic variability of *IPMK*, previously correlated to longevity can affect the susceptibility to LOAD.

2. Materials and Methods

Study population

For this study we analyzed 848 unrelated subjects born in Calabria (South Italy) and recruited across the whole territory through several campaigns focused on the monitoring of the quality of aging in the region. The sample included 568 healthy subjects aged 64-105 years (55 % females), and 280 patients with LOAD aged 77.8 ± 5.0 years (63% females). Their Calabrian ancestry was ascertained up to the third generation.

The group of health subjects was divided in two age classes according to two "thresholds of longevity", 88 years for men and 91 years for women, corresponding to the point after which a significant negative change in the slope of the survival curve of the Italian population occurs (Passarino et al, 2007). Males younger than 88 and females younger than 91 years will be defined as controls (N =309, mean age 74 years); in accordance with this, the rest of the sample, males older than 88 and females older than 91 years, will be here defined as long lived samples (N =259, mean age 96.9 years). In the long lived sample group females were 63%, while they were 49.5% in the adult controls. All subjects were free of the major age-related pathologies (e.g., cancer, type-2 diabetes, neurodegenerative and cardiovascular diseases), and were carefully assessed using a rigorous clinical history evaluation and a general/neurological examination, in order to exclude the presence of any neurological disorder.

LOAD patients were from the same geographical region and were enrolled by the Regional Neurogenetics Center (Lamezia Terme, Cz, Italy). Clinical diagnosis for LOAD was performed through the criteria of the National Institute on Aging, and the Alzheimer's Association workgroup (McKhann et al, 2011). All patients were fully characterized from a clinical point of view and a set of physical and biochemical parameters were measured. Cognitive status was investigated through Mini Mental State

Examination (MMSE) (Folstein et al, 1975). MMSE scores were adjusted for age and educational level according to Magni et al, 1996.

An informed written consent was signed by all subjects or their legal representative. This study was performed according to the Declaration of Helsinki with appropriate ethics committee approval.

SNP selection and genotyping

A panel of 17 SNPs within approximately 30 kb encompassing the entire *IP6K3* gene and its 5′ and 3′ flanking regions were genotyped in all subjects included in the study, and chosen based on those genotyped in the previous study by Crocco et al, 2016. Similarly, 14 SNPs were investigated for *IPMK*, mapping within and nearby the gene and prioritized by a tagging approach (De Rango et al, 2019). Genotyping was performed by iPlex Gold Genotyping Assay and Sequenom MassArray (Sequenom, San Diego, CA, USA) technology, following the manufacturer's instructions. SNP assays were designed using Sequenom's MassARRAY Assay Design v3.0 Software. Spectra were analyzed using MassARRAY Typer v3.4 Software (Sequenom). For quality control, to assess the reliability of the genotype identification protocols, about 10% of the samples were reanalyzed and the concordance rate of the genotypes was higher than 99%. For additional quality control, genotypes were excluded if Hardy-Weinberg equilibrium among controls $p < 0.05$ or call rates < 90%.

Linkage disequilibrium and functional annotation analysis of associated SNPs

Linkage disequilibrium analysis, LD, was explored using information from HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and considering SNPs associated with $r2 \geq 0.8$. To explore the potential function of the candidate SNPs, functional annotation analysis was performed by interrogating GTEx (Genotype–Tissue Expression) dataset (https://gtexportal.org/), a comprehensive survey of the functional consequences of genetic variation in non-coding regions at the transcript level from various human tissues samples (The Genotype-Tissue Expression (GTEx) Consortium, 2013). Positive or negative effects of the allele on the gene expression were estimated by considering the normalized effect size (NES) of the eQTLs, defined as the effect of the alternative allele (ALT) relative to the reference allele (REF) in the human genome reference GRCh38/hg38. For each gene indicated to be regulated by the candidate SNPs, a nominal p-value threshold of $p < 0.05$ was considered; top significant genes and relative tissues were finally included in the list of variant-gene pairs.

MDR analysis of epistatic interactions

For testing the epistatic interaction between pairs of SNPs, multifactor dimensionality reduction (MDR) was applied (Moore, 2004; Ritchie et al, 2001). This approach allows to estimate high-order interactions among genes collaborating with respect to a given phenotype and thus multilocus genotype combinations associated with high or low risk of disease. The entropy-based clustering algorithm used by MDR sets a contingency table for k SNPs and calculates case–control ratios for each of the possible multilocus genotypes. The MDR interaction model describes percentage of entropy (information gain or IG) by each factor (values in the nodes indicate independent main effect) or 2-way interaction. Graphical visualization is made through connections among the markers and help to interpret additive and non-additive interactions effects on phenotype: positive values of entropy indicate synergistic or non-additive interactions, while negative entropy values indicate redundancy between the markers or lack of any synergistic interaction between the markers.

For figures, networks were plotted by setting two-three-way combinations (Fig. 1a and 1b) and two-five-way combinations (Fig. 1S and 2S) of the attributes. Connections in red and orange indicate nonlinear or epistatic interactions, connections in green and brown indicate independence or additivity and redundancy (blue lines). For significance, permutation testing is applied, dividing the dataset into 10 portions, and using nine portions as a training data set, and the remaining as a testing data set. Missing genotypes were imputed with the MDR data tool software (version 0.4.3), by imputing the data from existing data set. MDR analyses were implemented in the open-source MDR software package version 3.0.2 (available on https://omictools.com/mdr-tool).

Statistical analyses

For each polymorphism, allele and genotype frequencies were estimated by gene counting from the observed genotypes. Hardy–Weinberg equilibrium was tested by Fisher's exact test. Pairwise measures of linkage disequilibrium (LD) between the analyzed loci was estimated by Haploview. (https://www.broadinstitute.org/haploview/haploview). The association between the analyzed genetic variants and the phenotypes under study was evaluated by logistic regression models. Different genetic models (dominant, additive and recessive) were used to test association, using for each SNP the minor allele as reference. For each SNP the most likely genetic model was then estimated on the basis of minimum level of statistical significance (Wald test p-value). For SNPs with rare homozygous genotype < 3%, only the dominant model was considered. Since SNPs were selected on the basis of prior

evidences of associations with the tested phenotypes, no Bonferroni correction was applied. Statistical analyses have been performed in R core environment (http://www.R-project.org/).

3. Results

Table 1 describes the sample groups analyzed in this study. These samples were previously tested for SNPs of *IP6K3* (adult control group vs LOAD patients), and for SNPs of *IPMK* (adult control group vs long-lived subjects). In the current study we checked the same groups for potential cross-phenotype associations, by testing the variants of *IP6K3*, previously studied in LOAD patients, also in long-lived subjects to compare them with the group of the adult control. Similarly, we studied the IPMK variants previously studied in long lived subjects also in LOAD patients to compare them with the group of adult control.

Analysis of the association of IP6K3 variants with Longevity

Results for the analyzed *IP6K3* variants are presented in Supplementary Table 1S. There was no association between sex and genotype frequency for any of the SNPs tested. Table 1 report the SNPs statistically associated with longevity in the present study, together with those associated with LOAD as reported by Crocco and colleagues (2016).

Table 1. Summary of the studied sample groups

	Subjects, n	Mean age (years, +/- SD)	Female, %
Adult control	309	74.06 ± 6.95	49.5
LOAD	280	77.8 ± 5.0	63
Long lived	259	96.92 ± 3.72	63

Our analysis found that the minor A allele of rs10947435 has a positive effect on longevity with a dominant model better fitting the data (OR=1.73, 95% CI 1.19-2.51; p=0.004). The additive model fit the data best for rs4713675 (OR=1.36, 95% CI 1.03-1.77; p=0.028), also with a positive effect of the minor T allele on longevity. As reported in Table 2, the same allele (A) of rs10947435 has been associated with an increased risk of LOAD, whereas no association has been detected between rs4713675 and LOAD risk (Crocco et al, 2016).

Table 2. Relevant comparisons of association results for *IP6K3* SNPs with longevity (present study) and LOAD (Crocco et al, 2016).

		Adult controls vs Long-lived (present study)			*LOAD vs Adult controls (Crocco et al, 2016)*		
IP6K3 SNPs	*Minor allele*	*OR*	*95% C.I.*	*pModel*	*OR*	*95% C.I.*	*pModel*
rs10947435	A	1.73	1.19-2.51	**0.004^{D}**	1.90	1.15-3.14	**0.010 ^{D}**
rs4713675	T	1.36	1.03-1.78	**0.028**^{A}	1.45	0.85-2.48	0.170 ^{D}
rs28607030	G	0.75	0.45-1.25	0.270^{R}	0.57	0.36–0.90	**0.011^{D}**

OR, odds ratio; CI, 95% confidence interval. OR adjusted for sex. PModel is the p-value of the best-fit genetic model. The choice of each genetic model was based on AIC value. D is dominant, R is recessive and ADD is the additive model.

Conversely, the variant rs28607030 did not result associated with longevity in this study, but instead has been associated with LOAD in the previous one. Hence, we observed either phenotype-specific (rs4713675 and rs28607030) or cross-phenotype (rs10947435) associations of SNPs with different direction of risk.

Analysis of the association of IPMK variants with LOAD

We then investigated the *IPMK* variants previously studied in relation to longevity (De Rango et al, 2019), in patients affected by LOAD. In consideration of the sex-specific effect of *IPMK* SNPs on longevity, the data were analysed according to gender. Similarly, to what was seen for longevity, we found SNPs significantly associated with LOAD in females, but not in males.

Complete results are reported in Supplementary Table 2S, whereas results for the SNPs associated significantly to one or both traits are summarized in Table 3.

Table 3. Relevant comparisons of association results for *IPMK* SNPs with LOAD (present study) and longevity (De Rango et al, 2019) in female samples.

		LOAD vs Adult controls (present study)			*Adult controls vs Long-lived (De Rango et al, 2019)*		
IPMK SNPs	*Minor allele*	*OR*	*95% C.I.*	*pModel*	*OR*	*95% C.I.*	*pModel*
rs2790156	A	0.25	0.06-0.92	**0.042^{R}**	*0.61*	*0.38–0.98*	**0.042**
rs2790234	G	0.52	0.27–0.99	**0.048^{D}**	*0.33*	*0.16-0.67*	**0.002**
rs2590320	A	0.23	0.06–0.83	**0.025^{R}**	0.57	0.36–0.91	**0.019**
rs6481383	T	0.96	0.55–1.47	0.697^{D}	0.59	0.37-0.94	**0.026**

rs1832556	A	0.40	0.13–1.19	0.101^{R}	0.59	0.37-0.94	**0.028**
rs2251039	T	0.61	0.39–0.95	**0.029^{D}**	0.61	0.38-0.97	**0.038**

OR, odds ratio; CI, 95% confidence interval. OR adjusted for sex. PModel is the p-value of the best-fit genetic model.The choice of each genetic model was based on AIC value. D is dominant, R is recessive and ADD is the additive model.

Out of the six SNPs negatively associated with female longevity, four SNPs, namely rs2790156, rs2790234, rs2590320 and rs2251039, conferred a protective effect on LOAD risk in the same gender ($p < 0.05$), with ORs (95% CI) of respectively 0.25 (0.06–0.92), 0.52 (0.27–0.99), 0.23 (0.06–0.83) and 0.61 (0.39–0.95). As before, we found cross-phenotype and phenotype-specific effects of SNPs.

We also performed haplotype analysis by including all the SNPs reported in Table 3. We identified the haplotype made of all the minor SNP alleles (namely A-G-A-t-a-T in Table 4) associated with reduced LOAD risk. The results also support a major effect of rs2790234-G allele on the trait; in fact, the A-G-A-t-a-T is significantly associated, while the A-C-A-t-a-T is not. Importantly, this is the same allele, lying in the six-SNPs haplotype, that exerts a major negative effect on longevity.

Table 4: Relevant comparisons of association results for IPMK haplotypes with LOAD (present study) and longevity (De Rango et al, 2019) in female samples.

	LOAD vs Adult controls (present study)			*Adult controls vs Long-lived (De Rango et al, 2019)*		
Haplotype	Frequency	Score	*P value*	Frequency	**Score**	*P value*
A-G-A-t-a-T	**0.073**	**-2.000**	**0.044**	**0.067**	**-2.897**	**0.002**
A-C-A-t-a-T	0.135	-1.144	0.252	0.138	-0.668	0.483
G-C-C-t-g-C	0.186	1.259	0.207	0.161	-0.353	0.715
G-C-C-c-g-C	0.566	-0.170	0.864	**0.616**	**2.155**	**0.024**

SNP-SNP interaction analyses

In view of the above findings, we evaluated SNP-SNP interactions both in LOAD and longevity of *IP6K3* and *IPMK* variants analyzed in the present and previous studies (Crocco et al, 2016; De Rango et al, 2019), by applying a Multi-Dimensional Reduction (MDR) method. In the interaction analysis, we included a SNP of *UCP4*, previously tested in the same sample groups, that negatively impacted on the

probability to attain longevity (Rose et al., 2011), while acted as a protective factor for LOAD (Montesanto et al., 2016).

This analysis has reported phenotype-specific SNP-SNP interactions, as shown in Supplementary Figures 1S and 2S, which plot the interaction network for longevity and AD, using one to five-way attributes combinations. As for longevity, the analysis also showed the contribution of sex as one of the factors influencing the entropy of the system.

For longevity, as shown by the entropy graph and dendrogram in Figure 1A and 1B, we found an epistatic interaction between rs2790234-*IPMK* and rs4713675-*IP6K3*.

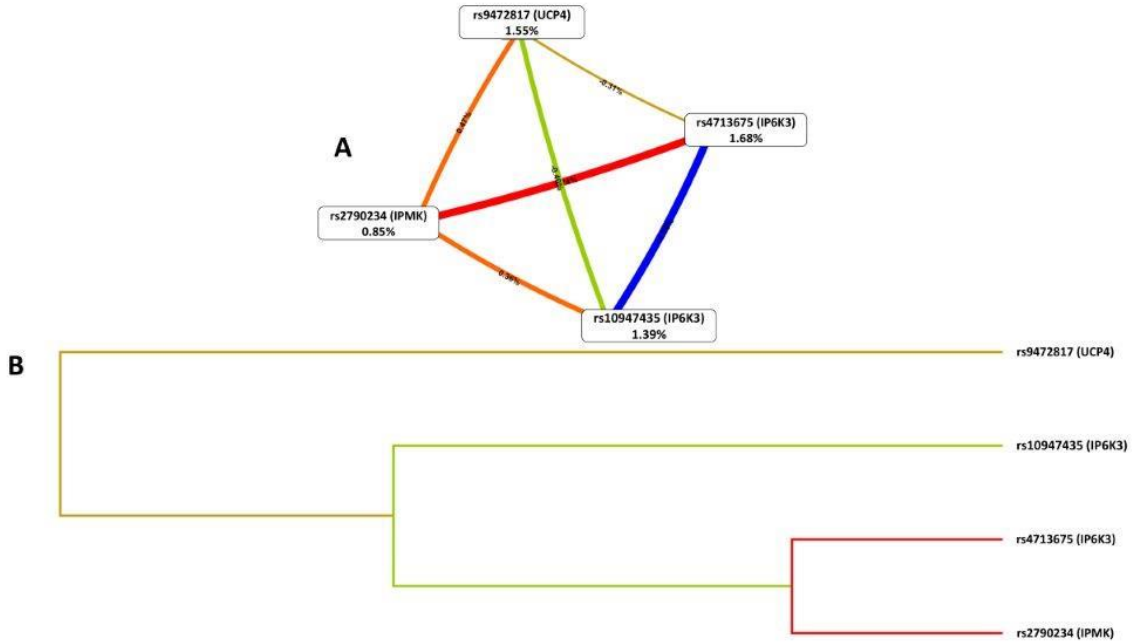

Figure 1. Interaction graph (a) and interaction dendrogram (b) in longevity data set, resulting from MDR analysis. In a, the network graph obtained by setting from two to three-way combinations of the attributes. For each SNP is reported in per cent the value of information gain (IG) and numbers in the connections indicate the entropy-based IG for the SNP pairs. Red bar and orange bar indicate the high-level synergies on the phenotype, while the brown indicate a medium-level interaction, green and blue connections with negative IG values indicate redundancy or lack of synergistic interactions between the markers. In b, the interaction dendrogram for the same dataset, obtained from the information gain values, organized in a distance matrix to carry out a hierarchical cluster analysis. Pairs of SNPs with stronger interactions have a smaller distance. The shorter is the line connecting two attributes, stronger is the interaction. As before, the color of the line indicates the type of interaction. Red and orange suggest there is a synergistic relationship (i.e. epistasis). Yellow suggests independence. Green and blue suggest redundancy or correlation.

As shown by red line, combining these two SNPs using MDR gives a positive information gain, evidence of an increased contribution to the phenotype respect to the single variants, which were in any

case associated to the phenotype. This interaction is significant and consistent (9/10 cross-validation consistency p<0.0001). Weaker interactions (orange lines) were found between rs2790234-*IPMK* and two SNPs, one being rs10947435-*IP6K3* and rs9472817-*UPC4*, both associated with longevity in single-SNP analysis (Rose et al, 2011). As for the blue line, such as for green one, these connections indicate a redundancy of the correspondent SNP pairs on the phenotype: this is particularly true here for rs4713675-*IP6K3* and rs10947435-*IP6K3*, which showed single associations with longevity.

In LOAD dataset, as shown by the entropy graph and dendrogram in Figure 2A and 2B, the analysis shows a strong single effect of rs9472817-*UCP4*, explaining alone the 9.28% of the entropy of the system; this SNP was previously reported to be associated with LOAD (Montesanto et al, 2016). This SNP epistatically interacts with rs1832556-*IPMK*, with a 7/10 cross-validation consistency and high training-balanced accuracy ($p < 0.0001$).

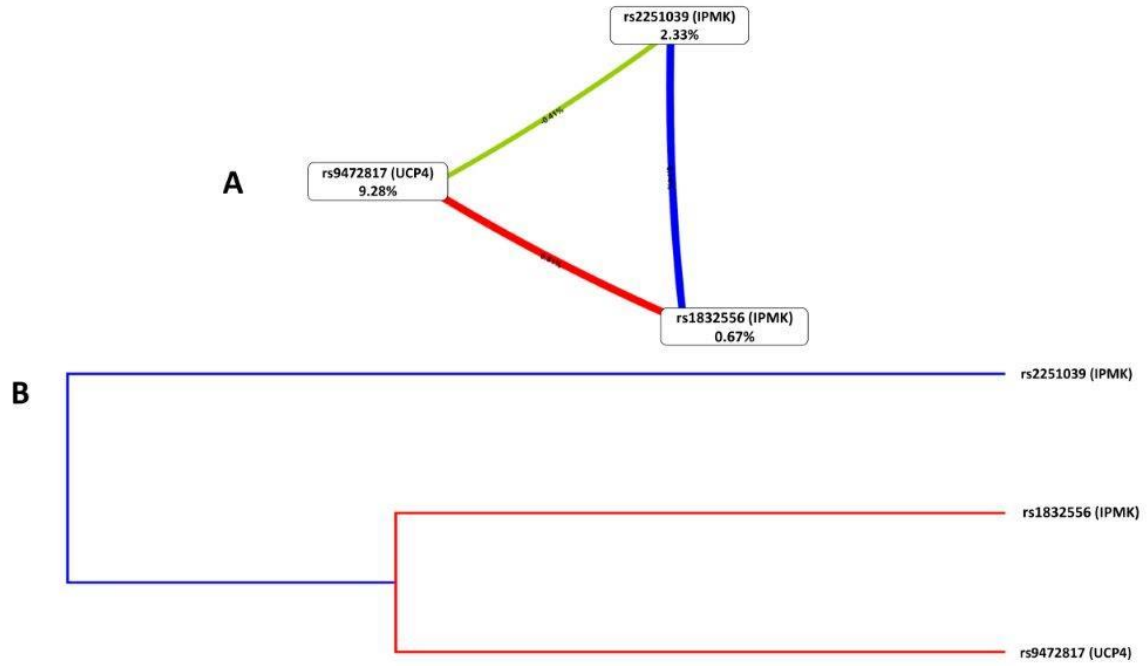

Figure 2. Interaction graph (a) and interaction dendrogram (b) in LOAD data set, resulting from MDR analysis. In a, the network graph obtained by setting from two to three-way combinations of the attributes. For each SNP is reported in per cent the value of information gain (IG) and numbers in the connections indicate the entropy-based IG for the SNP pairs. Red bar and orange bar indicate the high-level synergies on the phenotype, while the brown indicate a medium-level interaction, green and blue connections with negative IG values indicate redundancy or lack of synergistic interactions between the markers. In b, the interaction dendrogram for the same dataset, obtained from the information gain values, organized in a distance matrix to carry out a hierarchical cluster analysis. Pairs of SNPs with stronger interactions have a smaller distance. The shorter is the line connecting two

attributes, stronger is the interaction. As before, the color of the line indicates the type of interaction. Red and orange suggest there is a synergistic relationship (i.e. epistasis). Yellow suggests independence. Green and blue suggest redundancy or correlation.

Functional annotation of the associated SNPs

We performed functional annotation for better understanding the associations found with longevity and LOAD. We first queried the Haploreg database for SNPs in linkage disequilibrium (LD) (r^2 >= 0.8) with those significantly associated with our phenotypes (Table 3S in Supplementary Materials). For *IP6K3*, rs10947435 resulted in LD with SNPs in the same gene and with several markers of *UQCC2* (ubiquinol-cytochrome c reductase complex assembly factor 2, alias *MNF1*, Mitochondrial nucleoid factor 1); rs4713675 tags only another variant in the *IP6K3* gene, while rs28607030 is not in LD with other SNPs. As for *IPMK* markers, searches yielded all the significant SNPs in LD with a large number of variants in the same gene and with *CISD1* (CDGSH Iron Sulfur Domain 1, also termed *mitoNEET*) markers.
To further evaluate allele- and/or tissue-specific differences in gene expression we performed expression quantitative trait loci (eQTL) analysis by interrogating GTEX database. As supplementary Table 3S shows, this analysis reported both cis-regulatory and trans-regulatory allele-specific effects for all the tested SNPs; in particular, the analysis showed allele- and tissue- specific effects on genes involved in several cellular functions including mitochondrial activity.

1. Discussion

IP6K3 and *IPMK* gene products are crucial in the generation of inositol poly- and pyro-phosphates and, also, interact with other cellular components to control numerous aspects of cell metabolism in response to distinct cellular signals (Mukherjee et al, 2020; Kim et al, 2017; Wilson et al, 2013). Data from previous studies (Crocco et al 2016; De Rango et al 2019) show that *IP6K3* and *IPMK* loci harbour variants that are associated with LOAD and longevity phenotype, respectively. As many reports have highlighted that genetic variation affecting neurodegeneration may affect longevity as well (Nygaard et al, 2019), and vice versa, we investigated the variation of these genes for both phenotypes.
We found that an allele within *IP6K3* locus (rs10947435-A), previously reported to be associated with increased risk for LOAD (Crocco et al, 2016), increased the chance to become long-lived, while a subset of alleles at *IPMK* locus (rs2790156-A, rs2790234-G, rs2590320-A, rs2251039-T), that were found to decrease the chance to become long-lived (De Rango et al, 2019), decreased the risk for LOAD.
Several scenarios fit puzzling associations, which may implicate epistatic SNP-SNP interactions and/or different genetic mechanisms of pleiotropy, from the different forms of biological pleiotropy to spurious

pleiotropy (Hodgkin, 1998; Solovieff et al, 2013). Our interaction analysis showed phenotype-specific interactions, which reasonably could represent a potential factor contributing to the complex observed associations. Puzzling genetic associations may also represent the effects of co-localizing, that is causative, common or rare, variants at the same locus (or proximal loci) tagged by the same SNP due to linkage disequilibrium (LD). It is also worth noting that an allele may differently affect the expression of near (cis-eQTL) or distant (trans-eQTL) genes or even it may have different effects on different tissues, thus likely exhibiting context-specific effects with different phenotypic consequences. This could be particularly true for genes, such as *IP6K3* and *IPMK*, endowed with more than one molecular function, or participating in very different biological processes that could require the coordinate action of distinct signalling network.

The LD analysis performed, demonstrated that the associated SNPs are in LD not only with variants on the gene where they reside but also with those of other genes; thus, they may act as potential proxy markers for other SNPs in the same chromosomal region. Moreover, data extracted from GTEx database revealed allele-specific cis- and trans-eQTL effects across different tissues. We found that *IP6K3* rs10947435-A and rs4713675-T alleles also regulate, in a tissue specific manner, *BAK1* and *UQCC2* genes, both of which are related to mitochondrial function (Gross, 2016; Tucker et al, 2013). This is also the case for the associated *IPMK* variants, which are all cis-eQTL for *CISD1*, which encodes for a mitochondrial Fe-S protein, localized to the outer membrane of mitochondria and thought to play a role in regulation of mitochondrial lipid oxidation (Yuan et al, 2016). It is also quite interesting that the differential effects on gene expression of *IPMK* and *IP6K3* variants distinguish neuronal tissues with respect to the other tissues (see Table 3S).

Taken together, our result suggests that the biological basis of our observation may reside in mitochondria. The numerous evidences of the involvement of mitochondria in promoting neurodegeneration and in mediating longevity (Golpich et al, 2017; Rose et al, 2017) give plausibility to this hypothesis, that is supported by findings showing that inositol pyrophosphates act as energy sensors able to affect the cellular level of ATP, thus modifying the balance between mitochondrial oxidative phosphorylation and glycolytic flux (Gu et al, 2017; Szijgyarto et al, 2011). Besides, IPMK overexpression is reported to rescue deficits in mitochondrial metabolic activity in transgenic models of Huntington's disease (Ahmed et al, 2015) and also binds, in a glucose-mediated manner, the AMP-activated protein kinase (AMPK), a sensor of intracellular ATP levels that is rapidly activated after nearly all mitochondrial stresses (Bang et al, 2012). Additionally, IPMK appears to be a physiologic

cofactor of mTOR (Kim et al 2011), which regulates many aspects of mitochondrial function and one of the central modulators of lifespan (Wei et al, 2015). Further evidence, IP6K3 through the Inositol (1,4,5) Trisphosphate Receptor Type 3 (ITPR3) determines an increase of Ca^{2+} release from Endoplasmic Reticulum and a concomitant increase of the Ca^{2+} uptake in mitochondria (Càrdenas et al, 2010), which negatively affects the mitochondrial activity. Notably, besides affecting the *IP6K3* expression, the tested markers also differentially regulate the *ITPR3* gene expression (Supplementary Table 3S).

Remarkably, a behaviour like the one observed in the present study was reported for the polymorphism (rs9472817) of the uncoupling protein 4 gene (UCP4), a neuron specific mitochondrial membrane protein that uncouple biofuel oxidation from ATP. Indeed, the rs9472817-C allele was found to increase the risk of LOAD and the penetrance of APOE-ε4 allele (Montesanto et al, 2016), the risk for Frontotemporal Dementia (FTD) (Montesanto et al, 2018), and, at the same time, was found overrepresented among centenarians (Rose et al, 2011). Noteworthy, UCP4 has multifunctional properties on the neuronal system which include thermogenesis, neuronal plasticity, neuroprotection against oxidative stress, regulation of mitochondrial membrane potential and ATP level and Calcium homeostasis (Ramsden et al, 2012).

The highly orchestrated intracellular signaling pathways are often integrating and modulating mitochondrial physiology essential to the cellular metabolic and energetic needs. It therefore seems likely that mitochondria functions are important determinants of both diseases state and longevity. Mitochondria appear to be central hubs for neurodegeneration (Anderson et al, 2019). On the other hand, the central nervous system is significantly enriched with hubs (about 73% of the whole human interactome) among pathways which act at the crossroad of longevity and age-related disease networks (Wolfson et al, 2009). A further support to this hypothesis is given by the genetic variability of mtDNA, in particular by the 4336T>C mtDNA mutation which was found associated with Alzheimer's disease (AD) risk in a large number of studies and twice more frequent in ultra-nonagenarians than in younger controls (Brown et al, 1996; Santoro et al, 2010 and references therein).

Overall, these data support the idea that in neurodegenerative diseases and longevity, such as in other complex traits interactions may be more important than single polymorphic variations (Moore and Williams, 2002; Gilbert-Diamond and Moore, 2011) and that they play an important role on their non-additive heritability. In particular, interactions may account for the missing heritability of these traits and for their lack of replicability, as previously suggested on the basis of studies on model organisms (Mackay, 2014). On the other hand, it has been shown that using whole genome data to find interactions

affecting different phenotypes is extremely difficult due to huge number of interactions to be tested, the so called curse of dimensionality (Bellman, 1961; Gilbert-Diamond and Moore, 2011), which leads to great standard errors or to a reduction of power (Concato et al, 1993; Hosmer and Lemeshow; 2000; Freitas, 2001; Gilbert-Diamond and Moore, 2011). On the contrary, to highlight some polymorphisms which appear to be correlated to phenotypes, emerging with appreciable additive effects (Mackay, 2014), may represent a starting point for interaction analyses and functional studies which avoid a blind data mining.

2. Conclusions

Although additional large studies are warranted to validate our findings, some important conclusions emerge from this study. First, it supports a direct or indirect (i.e. mediate by the action of interacting partners) role of the multifunctional inositol-kinases IPMK and IP6K3 in both neurodegeneration and longevity, being crucial in inositol-mediated transduction pathways and metabolic routes essential for cell homeostasis and survival. Second, it supports the view that the contribution of a gene on the possibility to survive can be considered as an aggregated outcome of the multiple influences of its variants in the interactome network of the cell. Therefore, genes promoting longevity and/or affecting disease risks may be found in hubs interconnecting several signaling pathways, and their final effect will depend on the net effect of their interactions with other variants of proteins interacting with them.

The mtDNA variability may give a paradigmatic example of the complex relationships among alleles in different physiological contests. There is, in fact, evidence that while mutations in subunits of the complex I of the electron transport chain have a beneficial effect on longevity, the co-occurrence of mutations in complex I and III or in complex I and V seem to be detrimental (Raule et al, 2014). It is not surprising then that high frequency of mutations in complex I can be found in both mtDNA linked diseases (Man et al, 2004) and in long lived subjects (Tanaka et al, 1998).

Finally, from this study, especially if we put it in the context of previous studies, it emerges that mitochondrion represents a crucial crossroad for the functionality of the cell and, as the proteins involved in their functions interact with a number of other proteins which are in turn involved different pathways, the effect of each variant depends on other variants on different loci as well as on environmental factors.

References

1. Ahmed, I., Sbodio, J. I., Harraz, M. M., et al. Huntington's disease: Neural dysfunction linked to inositol polyphosphate multikinase. Proc Natl Acad Sci U S A. 2015;112(31):9751–9756.
2. Anderson, A. J., Jackson, T. D., Stroud, D. A., et al. Mitochondria-hubs for regulating cellular biochemistry: emerging concepts and networks. Open Biol. 2019;9(8):190126.
3. Azevedo, C., Saiardi, A. Eukaryotic Phosphate Homeostasis: The Inositol Pyrophosphate Perspective. Trends Biochem. Sci. 2017;42(3):219–231.
4. Bang, S., Kim, S., Dailey, M. J., et al. AMP-activated protein kinase is physiologically regulated by inositol polyphosphate multikinase. Proc Natl Acad Sci U S A. 2012;109(2):616–620.
5. Beekman, M., Nederstigt, C., Suchiman, H. E., et al. Genome-wide association study (GWAS)-identified disease risk alleles do not compromise human longevity. Proc Natl Acad Sci U S A. 2010;107(42):18046–18049.
6. Bellman, R. Adaptive Control Processes. Princeton, N. J.: Princeton University Press. 1961.
7. Brown, M. D., Shoffner, J. M., Kim, Y. L., et al. Mitochondrial DNA sequence analysis of four Alzheimer's and Parkinson's disease patients. Am. J. Med. Genet. 1996;61(3):283–289.
8. Cárdenas, C., Miller, R. A., Smith, I., et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell. 2010;142(2):270–283.
9. Concato, J., Feinstein, A.R., Holford, T.R. Ann Intern Med. 1993;118(3):201-210.
10. Crocco, P., Saiardi, A., Wilson, M. S., et al. Contribution of polymorphic variation of inositol hexakisphosphate kinase 3 (IP6K3) gene promoter to the susceptibility to late onset Alzheimer's disease. Biochim Biophys Acta. 2016;1862(9):1766–1773.
11. Dato, S., Rose, G., Crocco, P., et al. The genetics of human longevity: an intricacy of genes, environment, culture and microbiome. Mech Ageing Dev. 2017;165(Pt B):147–155.
12. De Rango, F., Crocco, P., Iannone, F., et al. Inositol Polyphosphate Multikinase (IPMK), a Gene Coding for a Potential Moonlighting Protein, Contributes to Human Female Longevity. Genes. 2019;10(2), 125.
13. Desfougères, Y., Wilson, M., Laha, et al. ITPK1 mediates the lipid-independent synthesis of inositol phosphates controlled by metabolism. Proc Natl Acad Sci U S A. 2019;116(49):24551–24561.
14. Fernandes, M., Wan, C., Tacutu, R., et al. Systematic analysis of the gerontome reveals links between aging and age-related diseases. Hum Mol Genet. 2016;25(21):4804–4818.
15. Field, A. E., Robertson, N. A., Wang, T., et al. DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. Mol cell. 2018;71(6):882–895.
16. Folstein, M. F., Folstein, S. E., McHugh, P. R. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. 1975;12(3):189–198.
17. Freitas, A.A. Understanding the crucial role of attribute interaction in data mining. Artif. Intel. Rev. 2001;16:177–199.
18. Freudenberg-Hua, Y., Freudenberg, J., Vacic, V., et al. Disease variants in genomes of 44 centenarians. Mol. Genet. Genomic Med. 2014;2(5):438–450.

19. Fu, C., Xu, J., Li, R. J., et al. Inositol Hexakisphosphate Kinase-3 Regulates the Morphology and Synapse Formation of Cerebellar Purkinje Cells via Spectrin/Adducin. J Neurosci. 2015;35(31):11056–11067.
20. Garagnani, P., Giuliani, C., Pirazzini, C., et al. Centenarians as super-controls to assess the biological relevance of genetic risk factors for common age-related diseases: a proof of principle on type 2 diabetes. Aging. 2013;5(5):373–385.
21. Gilbert-Diamond, D., Moore, J. H. Analysis of gene-gene interactions. Current protocols in human genetics. 2011;1:14.
22. Golpich, M., Amini, E., Mohamed, Z., et al. Mitochondrial Dysfunction and Biogenesis in Neurodegenerative diseases: Pathogenesis and Treatment. CNS Neurosci. Ther. 2017;23(1):5–22.
23. Gross A. BCL-2 family proteins as regulators of mitochondria metabolism. Biochim Biophys Acta. 2016;1857(8):1243–1246.
24. GTEx Consortium. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45(6):580-5.
25. Gu, C., Nguyen, H. N., Hofer, A., et al. The Significance of the Bifunctional Kinase/Phosphatase Activities of Diphosphoinositol Pentakisphosphate Kinases (PPIP5Ks) for Coupling Inositol Pyrophosphate Cell Signaling to Cellular Phosphate Homeostasis. J. Biol. Chem. 2017;292(11):4544–4555.
26. Hodgkin J. Seven types of pleiotropy. Int. J. Dev. 1998;42(3):501–505.
27. Hosmer, D.W., Lemeshow, S. Applied Logistic Regression. NY: John Wiley & Sons. 2000.
28. Kim, E., Ahn, H., Kim, M. G., et al. The Expanding Significance of Inositol Polyphosphate Multikinase as a Signaling Hub. Mol and cells. 2017;40(5):315–321.
29. Kim, S., Kim, S. F., Maag, D., et al. Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. Cell Metab. 2011;13(2):215–221.
30. Livermore, T. M., Azevedo, C., Kolozsvari, B., et al. Phosphate, inositol and polyphosphates. Biochem. Soc. Trans. 2016;44(1):253–259.
31. Lowsky, D. J., Olshansky, S. J., Bhattacharya, J., et al. Heterogeneity in healthy aging. J Gerontol A Biol Sci Med Sci. 2014;69(6):640–649.
32. Mackay T. F. Epistasis and quantitative traits: using model organisms to study gene-gene interactions. Nature reviews. Genetics, 2014;15(1):22–33.
33. Magni, E., Binetti, G., Bianchetti, A., et al. Mini-Mental State Examination: a normative study in Italian elderly population. Eur J Neurol. 1996;3(3):198–202.
34. McKhann, G. M., Knopman, D. S., Chertkow, H., et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011:7(3):263–269.
35. Montesanto, A., Crocco, P., Anfossi, M., et a. The Genetic Variability of UCP4 Affects the Individual Susceptibility to Late-Onset Alzheimer's Disease and Modifies the Disease's Risk in APOE-ε4 Carriers. J Alzheimers Dis. 2016;51(4):1265–1274.

36. Montesanto, A., Crocco, P., Dato, S., et al. Uncoupling protein 4 (UCP4) gene variability in neurodegenerative disorders: further evidence of association in Frontotemporal dementia. Aging. 2018;10(11):3283–3293.
37. Mooijaart, S. P., van Heemst, D., Noordam, R., et al. Polymorphisms associated with type 2 diabetes in familial longevity: The Leiden Longevity Study. Aging. 2011;3(1): 55–62.
38. Moore, J. H., Williams, S. M. New strategies for identifying gene-gene interactions in hypertension. Ann. Med. 2002;34(2):88–95.
39. Moore, J.H. Analysis of gene-gene interactions. Curr Protoc Hum Genet. 2004;1:1.14.
40. Moritoh, Y., Oka, M., Yasuhara, Y., et al. Inositol Hexakisphosphate Kinase 3 Regulates Metabolism and Lifespan in Mice. Sci. Rep. 2016;6:32072.
41. Mukherjee, S., Haubner, J., Chakraborty, A. Targeting the Inositol Pyrophosphate Biosynthetic Enzymes in Metabolic Diseases. Molecules (Basel, Switzerland). 2020;25(6):1403.
42. Nygaard, H. B., Erson-Omay, E. Z., Wu, X, et al. Whole-Exome Sequencing of an Exceptional Longevity Cohort. J Gerontol A Biol Sci Med Sci. 2019;74(9):1386–1390.
43. Park, J., Longo, F., Park, et al. Inositol polyphosphate multikinase mediates extinction of fear memory. Proc Natl Acad Sci U S A. 2019;116(7):2707–2712.
44. Passarino, G., Montesanto, A., De Rango, F, et al. A cluster analysis to define human aging phenotypes. Biogerontology. 2007;8(3):283–290.
45. Ramsden, D. B., Ho, P. W., Ho, J. W., et al. Human neuronal uncoupling proteins 4 and 5 (UCP4 and UCP5): structural properties, regulation, and physiological role in protection against oxidative stress and mitochondrial dysfunction. Brain and behav. 2012;2(4):468–478.
46. Raule, N., Sevini, F., Li, S., et al. The co-occurrence of mtDNA mutations on different oxidative phosphorylation subunits, not detected by haplogroup analysis, affects human longevity and is population specific. Aging cell. 2014;13(3):401–407.
47. Ritchie, M. D., Hahn, L. W., Roodi, N., et al. Multifactor-dimensionality reduction reveals high-order interactions among estrogen-metabolism genes in sporadic breast cancer. J. Hum. Genet. 2001;69(1):138–147.
48. Rojas, T., Cheng, W., Gao, Z., et al. Inositol hexakisphosphate kinase 3 promotes focal adhesion turnover via interactions with dynein intermediate chain 2. Proc Natl Acad Sci U S A. 2019;116(8):3278–3287.
49. Rose, G., Crocco, P., De Rango, F., et al. Further support to the uncoupling-to-survive theory: the genetic variation of human UCP genes is associated with longevity. PloS one. 2011;6(12):e29650.
50. Rose, G., Santoro, A., Salvioli, S. Mitochondria and mitochondria-induced signalling molecules as longevity determinants. Mech Ageing Dev. 2017;165(Pt B):115–128.
51. Santoro, A., Balbi, V., Balducci, E., et al. Evidence for sub-haplogroup h5 of mitochondrial DNA as a risk factor for late onset Alzheimer's disease. PloS one. 2010;5(8):e12037.
52. Sebastiani, P., Bae, H., Sun, F. X., et al. Meta-analysis of genetic variants associated with human exceptional longevity. Aging. 2013;5(9):653–661.
53. Solovieff, N., Cotsapas, C., Lee, P. H., et al. Pleiotropy in complex traits: challenges and strategies. Nature reviews. Genetics. 2013;14(7):483–495.

54. Szijgyarto, Z., Garedew, A., Azevedo, C., et al. Influence of inositol pyrophosphates on cellular energy dynamics. Science (New York, N.Y.), 2011;334(6057):802–805.
55. Tanaka, M., Gong, J. S., Zhang, J., et al. Mitochondrial genotype associated with longevity. Lancet (London, England). 1998;351(9097):185–186.
56. Thota, S. G., Bhandari, R. The emerging roles of inositol pyrophosphates in eukaryotic cell physiology. J Biosci. 2015;40(3):593–605.
57. Tsui, M.M., York, J.D. Adv Enzyme Regul. 2010;50(1):324-37.
58. Tucker, E. J., Wanschers, B. F., Szklarczyk, R., et al. Mutations in the UQCC1-interacting protein, UQCC2, cause human complex III deficiency associated with perturbed cytochrome b protein expression. PLoS genetics. 2013;9(12):e1004034.
59. Ukraintseva, S., Yashin, A., Arbeev, K., et al. Puzzling role of genetic risk factors in human longevity: "risk alleles" as pro-longevity variants. Biogerontology. 2016;17(1):109–127.
60. Wang M, Huang J, Liu Y, et al. COMBAT: A Combined Association Test for Genes Using Summary Statistics. Genetics. 2017;207(3):883-891.
61. Wei, Y., Zhang, Y. J., Cai, Y., et al. The role of mitochondria in mTOR-regulated longevity. Biol. Rev. Camb. Philos. Soc. 2015;90(1): 167–181.
62. Wilson, M. S., Livermore, T. M., Saiardi, A. Inositol pyrophosphates: between signalling and metabolism. Biochem. J. 2013;452(3):369–379.
63. Wolfson, M., Budovsky, A., Tacutu, R., et al. The signaling hubs at the crossroad of longevity and age-related disease networks. Int. J. Biochem. Cell Biol.2009;41(3):516–520.
64. Wundenberg, T., Mayr, G. W. Synthesis and biological actions of diphosphoinositol phosphates (inositol pyrophosphates), regulators of cell homeostasis. Biol. Chem. 2012;393(9):979–998.
65. Yokoyama, J. S., Wang, Y., Schork, A. J., et al. Association Between Genetic Traits for Immune-Mediated Diseases and Alzheimer Disease. JAMA Neurol. 2016;73(6):691–697.
66. Yuan, H., Li, X., Zhang, X., et al. CISD1 inhibits ferroptosis by protection against mitochondrial lipid peroxidation. Biochem Bioph Res Co. 2016;478(2):838–844.

Table 1S: Logistic regression analysis for the association between IP6K3 genotypes and longevity

SNP	*Minor Allele*	*Frequency (Controls =309)*	*Frequency (Cases=259)*	*OR*	*95% C.I.*	*pModel*
rs4713668	*T*	0.46	0.45	1.08	0.75-1.55	0.670^{D}
rs34252064	*A*	0.04	0.03	0.81*	0.40-1.62	0.550^{D}
rs12661400	*T*	0.22	0.20	0.91	0.64-1.30	0.610^{D}
rs622917	*T*	0.50	0.50	1.13	0.76-1.69	0.550^{D}
rs623813	*T*	0.13	0.14	0.52	0.14-1.88	0.310^{R}
rs545787	*A*	0.31	0.28	0.85	0.59-1.21	0.350^{R}
rs9469578	*T*	0.09	0.07	0.72*	0.40-1.30	0.270^{D}
rs16869463	*T*	0.09	0.07	0.81*	0.38-1.15	0.070^{D}
rs28607030	*G*	0.37	0.34	0.75	0.45-1.25	0.270^{R}
rs9469583	*T*	0.50	0.47	0.83	0.55-1.26	0.380^{R}
rs10947435	**A**	**0.39**	**0.46**	**1.73**	**1.19-2.51**	**0.004^{D}**
rs4713675	**T**	**0.45**	**0.53**	**1.36**	**1.03-1.78**	**0.028^{A}**

OR, odds ratio; CI, 95% confidence interval. OR adjusted for sex. Significant results in bold.

PModel is the p-value of the best-fit genetic model. The choice of each genetic model was based on AIC value. D is dominant model (risk in heterozygotes or minor allele homozygotes relative to common allele homozygotes), R is recessive model (risk in minor allele homozygotes relative to common allele homozygotes or heterozygotes), ADD is additive model (risk allele makes an equal (additive) contribution to the phenotype). *For these SNPs only the dominant model was considered since the rare homozygous genotype was <3%.

Table 2S: Results of the logistic regression models for IPMK SNPs in the female LOAD samples

SNP	*Minor Allele*	*Frequency (Controls =309)*	*Frequency (Cases=259)*	*OR*	*95% CI*	*pModel*
rs17636964	C	0.20	0.22	1.26	0.72–2.220	0.406D
rs12261547	C	0.03	0.01	0.59*	0.21–1.65	0.316D
rs2790156	**A**	**0.25**	**0.18**	**0.25**	**0.06–0.92**	**0.042R**
rs16911967	C	0.04	0.01	0.25*	0.05–1.22	0.088D
rs2790234	**G**	**0.12**	**0.06**	**0.52**	**0.27–0.99**	**0.048A**
rs11006086	C	0.10	0.12	0.53	0.25–1.11	0.096A
rs2590320	**A**	**0.27**	**0.12**	**0.23**	**0.06–0.83**	**0.019R**
rs6481383	T	0.41	0.41	0.96	0.55–1.47	0.697D
rs1832556	A	0.27	0.21	0.40	0.13–1.19	0.101R
rs11006100	A	0.16	0.18	0.86	0.25–2.92	0.814R

rs2251039	**T**	**0.26**	**0.16**	**0.61**	**0.39–0.95**	**0.029A**

Table 3S: Summary of functional annotation for relevant SNPs at *IP6K3* and *IPMK* loci, as obtained by Genotype-Tissue Expression (GTEx) pilot analysis (https://gtexportal.org/home/).

Gene	SNP	Minor Allele	Position	Effect on Longevity	Effect on LOAD	Variants in LD (Gene)	GTEX Allelic Effect size in Multiple tissues (P < 0.05)	
							Up-regulation	**Down-regulation**
IP6K3	rs10947435	A	5' utr	Favorable	Increased risk	19 (IP6K3) 14 (UQCC2)	***IP6K3*** (whole blood) ***TAP1*** (all tissues) ***TAP2*** (all tissues) ***UQCC2*** (brain, cerebellum, basal ganglia, skin)	***IP6K3*** (pancreas, esophagus, lung, stomach, skin, nerve, thyroid, heart) ***ITPR3*** (all tissues) ***BAK1*** (all tissues) ***UQCC2*** (thyroid, skeletal muscle, pancreas, spleen, heart, esophagus)
	rs4713675	T	5' utr	Favorable	Increased risk	1 (IP6K3)	***LEMD2*** (brain substantia nigra, testis, nerve, fibroblast) ***RPS18*** (colon, esophagus, stomach, thyroid) ***UQCC2*** (brain cerebellum, cerebellar hemisphere, basal ganglia, skin)	***IP6K3*** (all tissues) ***ITPR3*** (all tissues) ***BAK1*** (all tissues) ***LEMD2*** (esophagus, adrenal gland, thyroid) ***UQCC2*** (testis, pancreas, thyroid, skeletal muscle, spleen, skin, heart)
	rs28607030	G	5' utr	No effect	Decreased risk	No one	***IP6K3*** (brain basal ganglia, substantia nigra, nerve, thyroid, pancreas, esophagus, testis, skin, artery) ***ITPR3*** (whole blood, nerve, skin, spleen, pituitary, lung, colon) ***UQCC2*** (testis, heart, spleen, thyroid, esophagus)	***IP6K3*** (adrenal gland, salivary gland) ***ITPR3*** (brain cerebellum, cerebellar emisphere, fibroblast) ***BAK1*** (all tissues) ***LEMD2*** (all tissues) ***RPS18*** (all tissues) **UQCC2** (brain cerebellum, cerebellar hemisphere, basal ganglia, frontal cortex, skin)

IPMK	rs2790156	A	Intron 2	Unfavorable	Decreased risk	264 (IPMK) 40 (CISD1)	***IPMK*** (brain hippocampus, cerebellar emisphere, skin, testis, esophagus) ***CISD1*** (brain cerebellum, cortex, cerebellar emisphere, esophagus)	***IPMK*** (Whole blood, fibroblasts, adipose, spleen, heart) ***CISD1*** (nerve, whole blood, testis, spleen, colon, thyroid, esophagus, skin)
	rs2790234	G	Intron 3	Unfavorable	Decreased risk	23 (IPMK) 1 (CISD1)	***IPMK*** (esophagus, testis) ***CISD1*** (brain cortex, frontal cortex, basal ganglia, cerebellum, cerebellar emisphere, esophagus)	***IPMK*** (whole blood, colon, lung, fibroblast, heart, adipose) ***CISD1*** (whole blood, testis, colon, nerve, lung, thyroid, spleen)
	rs2590320	A	Intron 4	Unfavorable	Decreased risk	265 (IPMK) 39 (CISD1)	***IPMK*** (brain hippocampus, cerebellar emisphere, esophagus, skin, testis, pancreas) ***CISD1*** (brain cerebellum, cortex, cerebellar emisphere, esophagus)	***IPMK*** (Whole blood, fibroblast) ***CISD1*** (Whole blood, spleen, testis, fibroblast, nerve, lung)
	rs2251039	T	5’ utr	Unfavorable	Decreased risk	253 (IPMK) 39 (CISD1)	***IPMK*** (brain hippocampus, cerebellar emisphere, esophagus) ***CISD1*** (brain cerebellum, cortex, cerebellar emisphere, esophagus)	***IPMK*** (Whole blood, fibroblasts, heart) ***CISD1*** (whole blood, adipose, skin, nerve, testis, lung, colon)
	rs6481383	T	Intron 5	Unfavorable	No effect	5(IPMK) 2 (CISD1)	***IPMK*** (brain hippocampus, basal ganglia, cerebellar emisphere, esophagus,	***IPMK*** (Whole blood, fibroblast, heart) ***CISD1*** (whole blood)

							skin) ***CISD1*** (brain hippocampus, basal ganglia, cerebellar emisphere, esophagus)	
	rs1832556	A	5'utr	Unfavorable	No effect	210 (IPMK) 39 (CISD1)	***IPMK*** (brain hippocampus, cerebellar emisphere, skin, esophagus, testis) ***CISD1*** (brain cerebellum, cortex, cerebellar emisphere, esophagus)	***IPMK*** (Whole blood, adipose, fibroblast, heart) ***CISD1*** (whole blood, testis, skin, lung, spleen, thyroid, fibroblast)

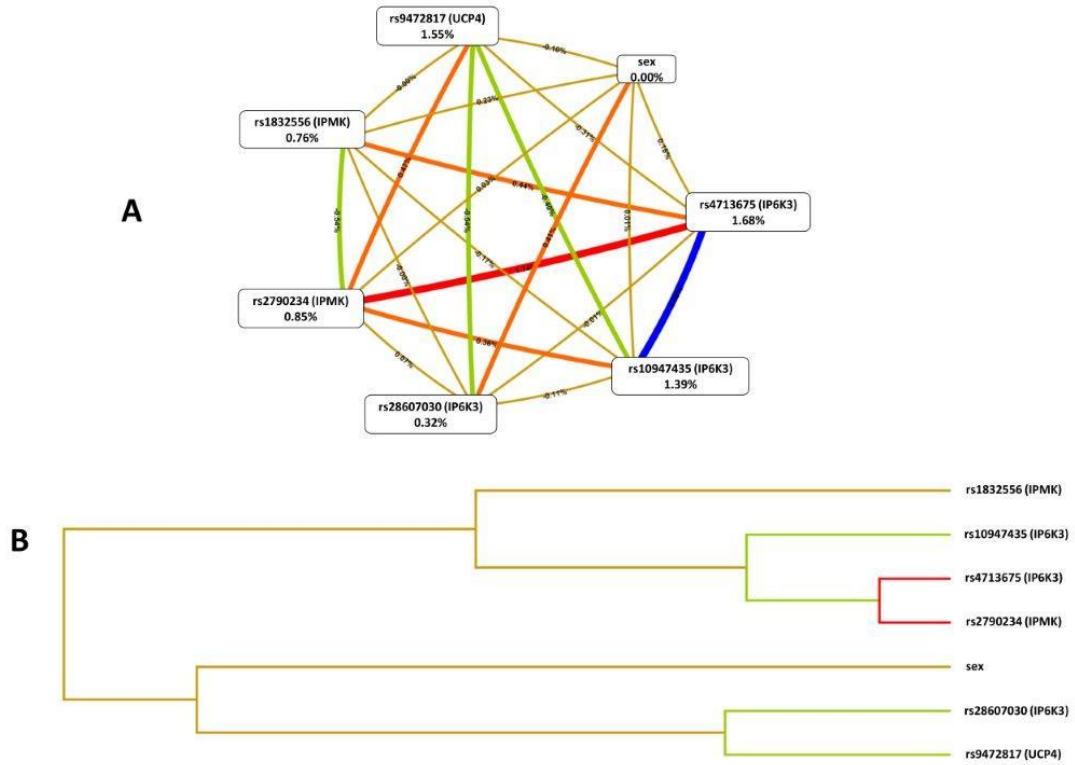

Figure 1S. Interaction graph (a) and interaction dendrogram (b) in longevity data set, resulting from MDR analysis. In a, the network graph obtained by setting from two to five-way combinations of the attributes. For each SNP is reported in per cent the value of information gain (IG) and numbers in the connections indicate the entropy-based IG for the SNP pairs. Red bar and orange bar indicate the high-level synergies on the phenotype, while the brown indicate a medium-level interaction, green and blue connections with negative IG values indicate redundancy or lack of synergistic interactions between the markers. In b, the interaction dendrogram for the same dataset, obtained from the information gain values, organized in a distance matrix to carry out a hierarchical cluster analysis. Pairs of SNPs with stronger interactions have a smaller distance. The shorter is the line connecting two attributes, stronger is the interaction. As before, the color of the line indicates the type of interaction. Red and orange suggest there is a synergistic relationship (i.e. epistasis). Yellow suggests independence. Green and blue suggest redundancy or correlation.

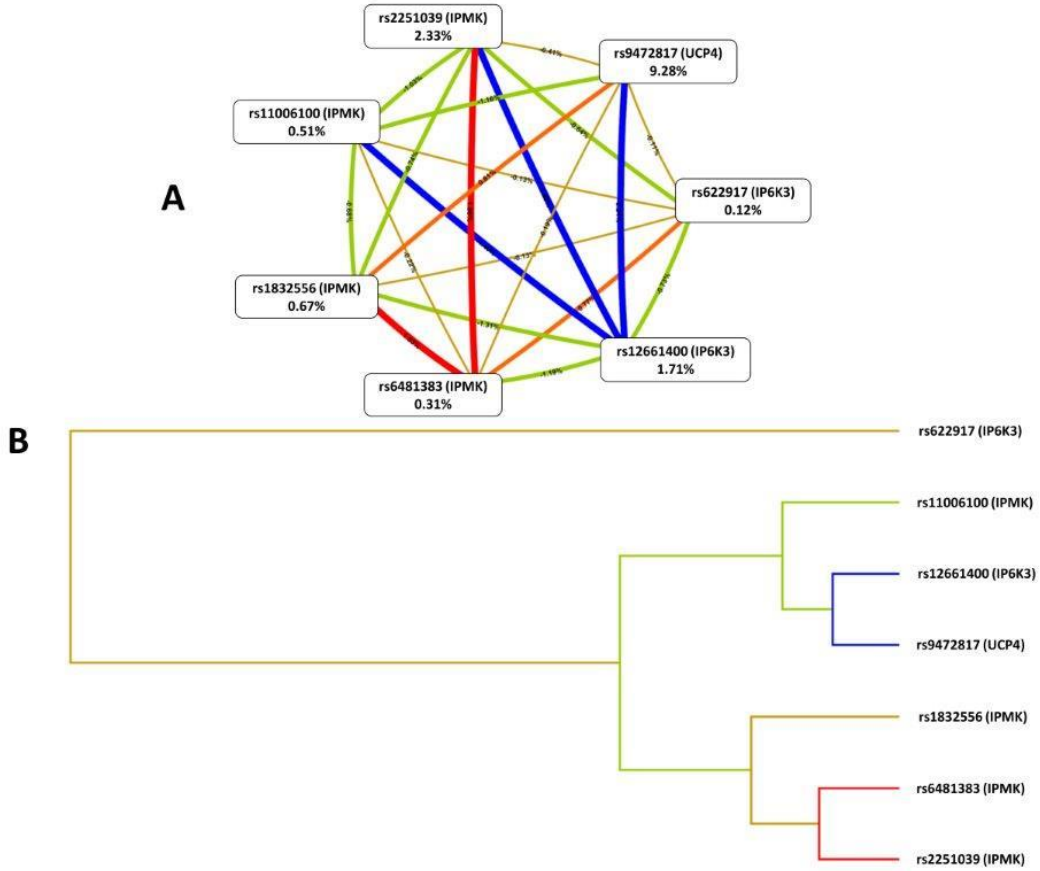

Figure 2S. Interaction graph (a) and interaction dendrogram (b) in LOAD data set, resulting from MDR analysis. In a, the network graph obtained by setting from two to five-way combinations of the attributes. For each SNP is reported in per cent the value of information gain (IG) and numbers in the connections indicate the entropy-based IG for the SNP pairs. Red bar and orange bar indicate the high-level synergies on the phenotype, while the brown indicate a medium-level interaction, green and blue connections with negative IG values indicate redundancy or lack of synergistic interactions between the markers. In b, the interaction dendrogram for the same dataset, obtained from the information gain values, organized in a distance matrix to carry out a hierarchical cluster analysis. Pairs of SNPs with stronger interactions have a smaller distance. The shorter is the line connecting two attributes, stronger is the interaction. As before, the color of the line indicates the type of interaction. Red and orange suggest there is a synergistic relationship (i.e. epistasis). Yellow suggests independence. Green and blue suggest redundancy or correlation.

CHAPTER 5

LAV-*BPIFB4* associates with reduced frailty in humans and its transfer prevents frailty progression in old mice

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Introduction

Frailty is a clinically recognizable state of increased vulnerability to stressor events resulting from the systemic decline in function and physiological reserve mechanisms with aging [1]. This weakening condition detrimentally affects the normal physical activity and is associated with an increased risk for adverse clinical outcomes and death [2]. Therefore, frailty reflects the individual's biological age and life expectancy better than chronological age [3]. Studies in long-living individuals (LLIs), which, in spite of their exceptional biological age, are protected from and cope better with age-related diseases, confirm this concept [4]. Moreover, several genetic factors that are reportedly implicated in the determination of exceptional longevity are also inversely related with frailty disabilities [5, 6].

The Bactericidal/Permeability-Increasing Fold-Containing Family B member 4 (*BPIFB4*) gene encodes a secreted protein, initially found to be expressed in salivary glands, and more recently discovered to play important pathophysiological roles at systemic level. A genome wide association study (GWAS), performed on an Italian set of LLIs and controls and validated on two independent populations from Germany and USA, identified the *BPIFB4* variants associate with lifespan [7]. We found a consistent enrichment of the minor allele of the nonsynonymous single nucleotide polymorphism (SNP) rs2070325 of *BPIFB4* (identifier: P59827.2), under recessive model, in LLIs. The rs2070325 is part of a four SNPs haplotype that codifies for a wild type variant (WT), a longevity-associated variant (LAV) and a rare variant (RV) of *BPIFB4*, represented respectively by the 66%, the 29.5% and the 4% of the alleles [7]. In more detail, the rs2070325 variation (Ile229Val) of *BPIFB4* is in perfect linkage disequilibrium with rs2889732 (Asn281Thr), while both show a limited amount of recombination events with rs11699009 (Leu488Phe) and rs11696307 (Ile494Thr). Thus, the main three alternative haplotypes are WT (Ile229/Asn281/Leu488/Ile494-BPIFB4 isoform), LAV Val229/Thr281/Phe488/Thr494-BPIFB4 isoform), and RV (Ile229/Asn281/Phe488/Thr494-BPIFB4 isoform) that carries the major alleles of rs2070325 and of rs2889732 and the minor allele of rs11699009 and rs11696307.

The BPIFB4 protein is expressed in undifferentiated and highly proliferative cells and in fetal/stressed heart tissue (cardiac hypertrophy), which share a common hypoxic environment. Overexpression of BPIFB4 isoforms induced the activation of stress response-related heat-shock proteins (HSPs) and the modification of protein homeostatic processes (translation, ribosome biogenesis, spliceosome), two processes that are usually lost during aging. Furthermore, the circulating levels of immunoreactive BPIFB4 protein are reportedly higher in healthy LLIs than in diseased LLIs or young controls [8]. Similarly,

$CD34^+$ hematopoietic cells and mononuclear cells (MNCs) of LLIs expressed higher levels of BPIFB4 than corresponding cells of young controls [8, 9]. Studies in experimental models of cardiovascular disease confirmed that overexpression of the human *LAV- BPIFB4* gene results in attenuation of hypertension, atherosclerosis, and ischemic disease, which are hallmarks of aging [4].

The aim of the present study was to investigate the novel hypothesis that *BPIFB4* haplotypes segregate with frailty, which was assessed using a methodology specifically developed for the geographical location of the study [10]. We challenged this hypothesis in a cohort of elderly subjects with an age comprised between 65-90 years, a life period where frailty is acknowledged to increase progressively in humans [2]. In addition, to obtain direct functional evidence for this association, we attempted to combat frailty in old mice using gene therapy with *LAV-BPIFB4*. Among various assessment tools for frailty in mice [11–14], we have chosen to use an index that calculate the accumulation of deficits [14] and we also validated the results considering treatment outcomes in a combined model comprising physical frailty [11] and mortality. Results of this research highlight the predictive value and therapeutic potential of *LAV-BPIFB4* in age-related frailty.

Results

Association with frailty and survival in humans

The baseline characteristics of the cohort are illustrated in Table 1.

Table 1. General characteristic of the analyzed groups at the time of the recruitment.

Calabrian cohort	**(N= 237)**
Mean Age (SD)	73.4 (6.2)
Age Range	65–90
Female, N (%)	131(55.3)
Non-Frail, N (%)	121 (51.0)
Frail, N (%)	116 (49.0)

The association analyses with frailty trait showed that the LAV homozygous haplotype is under-represented in frail subsets of the cohort ($p = 0.030$ vs. other haplotypes), thus suggesting a potentially protective role of this variant (Table 2 and Figure 1). Conversely, carriers of the RV haplotype are more frequently frail ($p = 0.031$ vs. other haplotypes), whereas the WT haplotype did not allow to distinguish between frail and not frail subjects (Table 2 and Figure 1).

Table 2. Distribution of BPIFB4 haplotypes in Calabria population stratified by frailty levels.

Haplotype	Models	Non frail N (%)	Frail N (%)	p-value*
LAV	Homo Carriers/Others	13 (10.7) 108 (89.3)	4 (3.4) 112 (96.6)	**0.030**
RV	Carriers Others	7 (5.8) 114 (94.2)	16 (13.8) 100 (86.2)	**0.031**
WT	Homo Carriers/ Others	53 (43.8) 68 (56.2)	48 (41.4) 68 (58.6)	**0.403**

*p-value assessed by Fisher's Exact test.

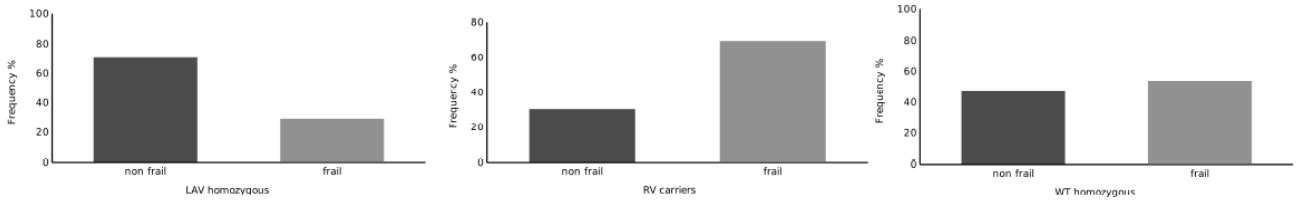


Figure 1. Distribution of RV carriers, LAV Homozygous and WT Homozygous subjects across the groups defined by cluster analysis.

Looking at the variants influence on lifespan, we analyzed the survival of RV and LAV carriers using a Cox regression. As reported in Figure 2, we could see a negative effect on survival by the RV haplotype (adjusted HR = 4.066; p = 0.044) but not by the LAV haplotype (adjusted HR = 0.002; p = 0.97). Likewise, the WT haplotype was uninfluential (data not shown).

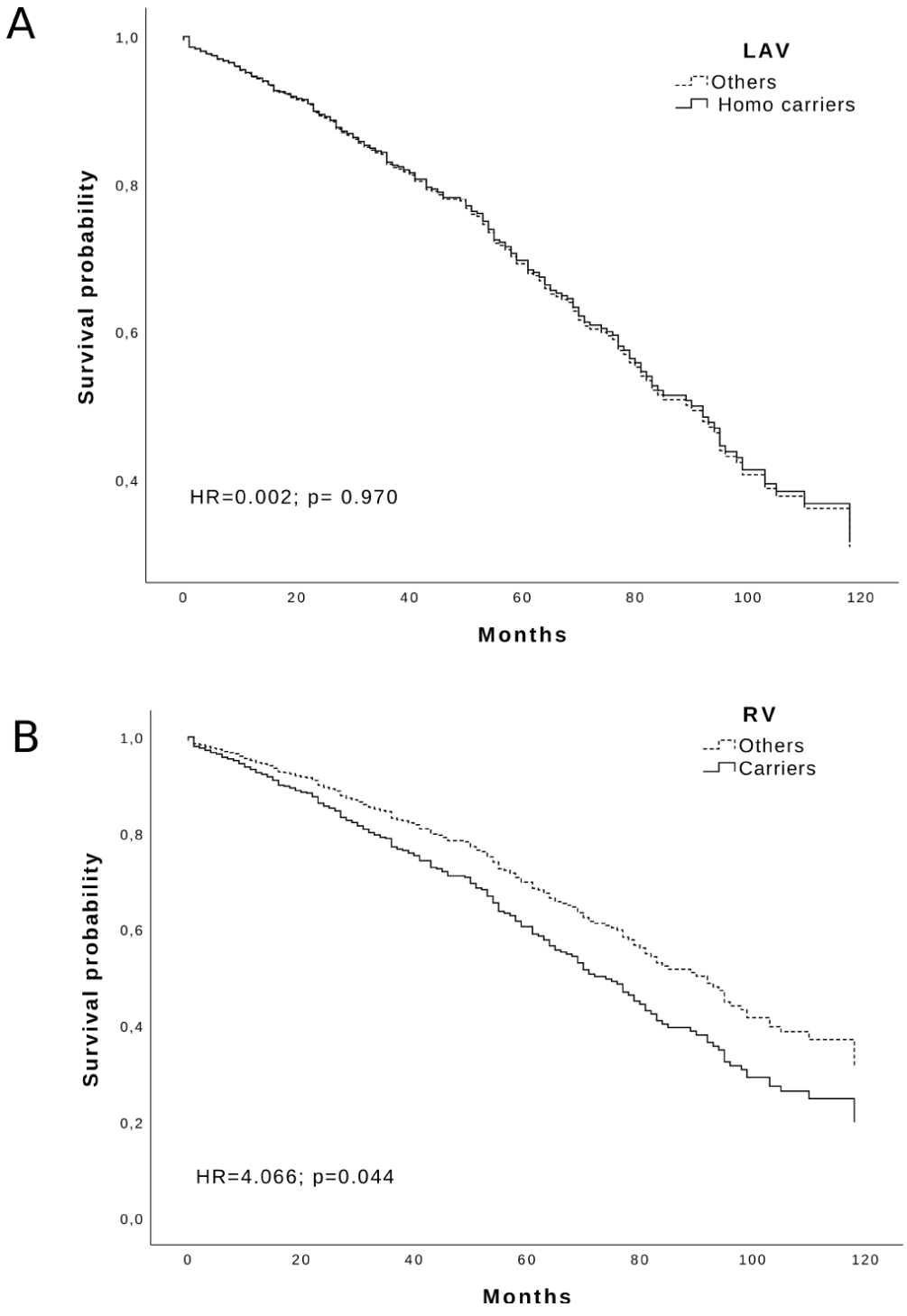


Figure 2. Survival function of: (**A**) LAV Homozygous carriers and (**B**) RV carriers (solid line) vs others (dotted line) in the Calabria cohort. Time is expressed in months, where 0 is considered the time of recruitment, and each individual is followed up for survival status till death. Adjusted HR and p-values are reported inside the Figure.

Effect of gene therapy with AAV-LAV-*BPIFB4* on frailty in aging mice

Systemic gene therapy with LAV-*BPIFB4* resulted in a slight but significant delay in the progression of clinical frailty. In fact, mice injected with LAV-*BPIFB4* displayed a significant lower frailty index at 7-month follow-up (2 months after the last injection of the gene) as compared with controls (Figure 3A). A subgroup analysis by age groups revealed that only old mice, but not adult mice, treated with LAV-*BPIFB4* had a lower frailty index from 5- to 7-month follow-up compared with age-matched controls (Figure 3B). On the other hand, an analysis based on the prevalence of physical frailty could not capture a significant effect of LAV-*BPIFB4* gene therapy in old mice at the 7-month assessment (20.0 vs. 44.4% in controls, p = 0.170 by Fisher's exact test, Supplementary Table 1).

From the time of the first injection (3rd month) up to the 12th month assessment, we recorded 24 deaths in controls and 22 deaths in the LAV-*BPIFB4*-treated group within the old cohort, while the deaths in the adult cohort were 10 and 11, respectively. We found no significant difference in the mortality hazard between the treatment and control groups at the 12th month, either considering the whole population (control vs. treatment group HR = 1.33, CI = 0.75-2.34; p = 0.33) or the old sub-population (control vs. treatment group HR = 1.28, CI = 0.68-2.41; p = 0.43).

We deemed that any further follow-up for survival after the 12th month would have been unnecessary as the colony of old mice was numerically exhausted. We also argued that the excess death from the 7-month assessment onwards could had invalidated the power of the physical frailty analysis. Since part of these deaths in the old group may have arisen as an outcome of frailty, we also compared the combined prevalence of physical frailty and deaths in the treated and control mice. Accordingly, the prevalence of this combination was significantly lower in LAV-*BPIFB4*-treated old mice as compared with control mice (28.6 and 61.5%, respectively, p = 0.03 by Fisher's exact test, Table 3).

Table 3. Prevalence of combined physical frailty and deaths1 in treated and control mice at the 3rd month (before treatment) and at the 7th month (after treatment) from the inclusion in the study*.

Age of mice at the beginning of the study	Status	Month 3 (Before treatment)		Month 7 (Post treatment)	
		Control	LAV-*BPIFB4*	Control	LAV-*BPIFB4*
Adult mice	Frail + deaths	2 (8.3%)	1 (4.5%)	5 (20.8%)	2 (9.1%)
	Non-frail	22 (91.7%)	21 (95.5%)	19 (79.2%)	20 (90.9%)
Old mice	Frail + deaths	4 (15.4%)	3 (10.7%)	16 (61.5%)	8 (28.6%)
	Non-frail	22 (84.6%)	25 (89.3%)	10 (38.5%)	20 (71.4%)

[1] Death-events included in the outcome consists of all mice dying from month 3 up to month 7.
* Data are reported as number of mice (%). For adult mice all comparisons between treated and control groups were not significant ($p > 0.05$). For old mice at month 3, $p = 0.741$ by Fisher's exact test; For old mice at month 7, $p = 0.03$ by Fisher's exact test.

Interestingly, the proportion of old mice with reduced grip strength and gait disorders, which are parameters of the clinical frailty index related to the physical phenotype, were improved at the 7th month by LAV-*BPIFB4* (Supplementary Table 2).

Discussion

Frailty is a common clinical syndrome of functional decline related to aging characterized by marked vulnerability. Its distinctive phenotype can be categorized on physical attributes, such as stamina, strength, speed, activity and weight, [15] or as a deficit model, in which the risk of adverse events accumulates due to the impairment in several psychophysical domains [16]. The latter definition appears to be better suited to predict mortality both in humans [17] and mice [18] but there is no current consensus about frailty assessment tool that should be used.

There is a remarkable heterogeneity for frailty in different geographic areas. Therefore, we used a frailty index tool that was previously employed in the same region of our study to foresee the health status and perspective survival of a geriatric population of 680 subjects with an age range of 65–108 years [10]. This classification was replicated in two large longitudinal Danish samples, which confirmed the predictive soundness after 10-years of follow up [19]. The analysis revealed a significant underrepresentation of frailty in old individuals of the homozygous LAV-*BPIFB4* haplotype. Moreover, we observed a reduced survival rate in RV carriers during 10-years follow-up as compared with the carriers of the LAV and WT haplotypes.

To validate the cause-effect value of the gain-of-function mutation, we delivered the human LAV-*BPIFB4* gene to adult or old mice via a viral vector. Interestingly, in old mice, gene therapy attenuated the progression of clinical frailty, whereas the treatment was not effective on physical frailty. Mice develop physical disability only at the extreme stage of life, as seen in longitudinal screening investigations [20]. Therefore, one possible explanation for the lack of physical benefit by LAV gene therapy is that there was little room for improvement at the age studied here. Moreover, the physical frailty phenotype not only underlies a different form of vulnerability compared with clinical frailty, but also requires larger sample sizes. Considering the number of mice lost to follow-up due to premature death, the assessment on physical frailty at 7th months might not have enough power to reject the null hypothesis. To mitigate this limitation, we considered the effect of gene therapy on the combined outcomes of physical frailty and death events. Using this approach, we

found a significant improve-ment in LAV-treated animals compared with age- matched controls. Hence, the data support a protective role of the gene therapy in the onset of clinical and physical frailty in old rodents.

Observational studies have linked endothelial dysfunction with frailty, thus supporting the concept that poor circulation could compromise the whole body homeostasis and thereby the ability of an old organism to cope with stress [21, 22]. Previous studies of *BPIFB4* gene transfer in elderly mice demonstrated the LAV exerts benefits on endothelial function, while RV is detrimental. This dichotomy corresponded to consensual changes in eNOS activity, which was increased by LAV and reduced by RV [9, 23]. Therefore, one possible interpretation of the new data presented here is that LAV can halt frailty by protecting the vasculature from aging and aging-related risk factors, whereas RV causes the contrary.

Both atherosclerosis and inflammatory processes have been considered as central hubs for frailty [24, 25]. A systematic review and meta-analysis suggested that frailty and pre-frailty are associated with higher inflammatory parameters [26]. We recently showed that LAV-*BPIFB4* gene therapy counteracted the development of vascular atherosclerosis in ApoE knockout mice fed a high fat diet [27]. Moreover, LAV-*BPIFB4* protein induced M2 monocytes polarization and exerted anti-inflammatory effects [28]. Therefore, it is tempting to speculate that LAV-*BPIFB4* may have contrasted the low-grade chronic inflammation that is typical of progressive atherosclerotic disease.

The association of the LAV haplotype with lower frailty in elderly subjects and the reduced frailty observed in mice treated with LAV-*BPIFB4* gene therapy are in perfect agreement. However, in both cases (human and mice), there was no impact of LAV on survival. In the human study, however, the RV haplotype was associated with a worse survival. This is not the first case where interventions influencing health span do not benefit lifespan. There are, indeed, evidence from studies in animal models showing that genetic or other types of intervention, such as life-long spontaneous exercise [29] and supplementation with nicotinamide [30], improve aspects of healthy aging, without concomitantly increasing lifespan. This might occur, for instance, if an intervention modulates age-dependent disorders that are cause of disability and morbidity but are not the principal causes of mortality. Likewise, tissue-specific effects of genetic variations might improve the effects of aging in an organ without improving survival. In addition, the lack of association of the LAV haplotype with survival in the human cohort may be attributed to a lower penetrance on this trait. Therefore, the follow-up time of 10 years on a small population may not be long enough to detect variations in the risk of death. This would not be the case for the RV haplotype, which is rarest but likely more penetrant on the survival phenotype. The enrichment of the LAV haplotype we have previously reported in LLIs could be indeed the result of a higher mortality of RV carriers.

Study limitations

Although genetic and molecular evidences support the role of *BPIFB4* haplotypes in aging and longevity, additional studies should be carried out to confirm their role in the susceptibility to frailty. Due to the specificity of the studied cohort, replication in different and larger populations should be performed. Furthermore, an evaluation for a longer time is necessary to definitively determine the impact of the LAV haplotype on the risk of death. Likewise, larger cohorts of mice would be necessary to provide enough power in the assessment of survival.

Conclusions

To the best of our knowledge, this is the first study presenting both associative clinical evidence and experimental proof of concept for a gene's haplotypes to influence frailty. These data could have important clinic and therapeutic implications. Screening the *BPIFB4* haplotypes could provide important information on the individual's risk to develop disability with aging and thus help clinicians in elaborating precision medicine decisions. This and similar genome-based technologies could shift the treatment (and associated costs) from acute intervention and disease management to an effort in assessing health and proactive control of disease risks and prevention. Furthermore, preclinical data on *BPIFB4* gene therapy provide a further scope for the horizontal transfer of the healthy features of centenarians to individuals at risk. Clinical studies confirming safety and efficacy of such therapy could pave the way to new treatment capable of improving general health and reducing care costs dramatically.

Methods

Human sample description

The Calabria cohort involved in this study is a subset of a larger population already described by Montesanto et al [10]. This subset includes a total of 237 unrelated individuals (106 men and 131 women) 65–90 years old (median age 72 years), participated in the present study. All the subjects lived in Calabria (southern Italy) and their origin in the area have been verified up to the grandparent's generation, as previously described [10]. Health status was ascertained by medical visit carried out by a geriatrician through a structured interview including physical and cognitive tests, as well as questions on common diseases occurred in the past. At the same time, it was performed DNA extraction and hematological analyses on peripheral venous blood samples.

For analyzing the correlation with quality of aging of the genetic variants investigated, we used the frailty classification of this sample, as obtained in a previous work [10]. In brief, according to this approach, each individual can be classified respect to his/her frailty level, determined by applying a

hierarchical cluster analysis (HCA) on specific geriatric parameters, including Mini Mental State Examination (MMSE), Self-Reported Health Status (SHRS), Activity of Daily Living (ADL) and Hand Grip (HG) strength. For this population, two clusters were considered: non frail (the cluster with subjects showing the best scores for the classification variables) and frail (the clusters with subjects showing the worst scores for the classification variables). Furthermore, Calabria cohort has been followed-up for 10 years.

Ethics statement

Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board. Each subject, before the visit, signed an informed consent, for the permission to collect blood samples and usage of register-based information for research purposes

Genotyping

Samples were genotyped using Taqman assays for SNPs rs2070325 and rs11699009, to identify haplotypes. Alleles of rs2889732 and rs11696307 were imputed, given that are in total LD with the previous named respectively. It was performed data analysis with QuantStudio software 1.1 (ThermoFisher Scientific).

Gene therapy with AAV-LAV-BPIFB4 in mice

Constructs and vectors used in this study

We used LAV-*BPIFB4*- and green fluorescent protein–encoding adeno-associated viral vectors (AAV serotype 9 with a TBG promoter) to transduce mice. Details on the construction of these constructs have been previously described [9].

Animal study

All experiments were performed according to the European Community Council Directives of 2010/63/UE and the protocol was approved according to current Italian law (D.Lgs. n. 26/2014) by the General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health with the authorization n° 130/2018-PR. We used C57BL/6J mice housed under specific pathogen-free (SPF) conditions in a room with controlled temperature (22 ± 2°C) and a 12-h light–dark cycle, with ad libitum access to food and water.

A total of 103 mice (71 males and 32 females) were used in the study. Three mice died before any treatment was performed and were excluded from the study. The mice were assigned to two age-matched experimental groups: a treatment group (AAV-LAV-*BPIFB4*; 50 mice) and a control group

(AAV-GFP; 50 mice). The experimental groups were further subdivided into 4 subgroups based on the age of the mice at the start of the study. The first group consisted of "adult controls" (24 mice, 11 females and 13 males) aged 16-17 months (mean age ± SD = 16.8 ± 0.7 months). The second group consisted of "treated adults" (22 mice, 9 females and 13 males) aged 16-17 months (mean age ± SD = 16.7 ± 0.7 months). The third group consisted of "old controls" (26 mice, 4 females and 22 males) aged 18-23 months (mean age ± SD = 21.6 ± 1.9 months). The fourth group consisted of "treated old" (28 mice, 8 females and 20 males) aged 18-23 months (mean age ± SD = 21.0 ± 2.0 months). We performed non-invasive measurements of clinical frailty once a month in all mice. Physical frailty data were recorded at the 3rd and at the 7th month from the start of the study. After recording the frailty data at the 3rd month, we injected (i.v.) into the tail vein 1*1014 viral particles of AAV-LAV-*BPIFB4* or AAV-GFP in the treatment and control groups, respectively. The same treatment was repeated at the 5th month from the start of the study. We also recorded the time to death for each mouse until the 12th month since the beginning of the experiment. Mortality occurred when animals died suddenly or euthanized due to illness. A detailed design of the study is reported in Supplementary Figure 1.

Measurement of frailty in mice

We measured both clinical and physical frailty in mice. We measured the clinical frailty index (FI) in mice based on the validated murine clinical FI tool described previously [14]. Details on the measurement of weight, body surface temperature and grip strength (which are included in the FI tool) have been also described previously [12]. FI data were recorded the second week of each month from 10 am to 2 pm. All measurements of frailty were performed within the SPF animal facility of INRCA in a dedicated area. The clinical FI score for each mouse was calculated using the checklist published previously [14]. Clinical assessment included evaluation of the integument, musculoskeletal system, vestibulocochlear and auditory systems, ocular and nasal systems, digestive system, urogenital system, respiratory system, signs of discomfort, as well as the body weight and body surface temperature. For each parameter, a score of 0 was given if there was no sign of a deficit, a score of 0.5 denoted a mild deficit and a score of 1 indicated a severe deficit. Deficits in body weight and body surface temperature were scored based on their deviation from average reference values obtained from the entire cohort. Values that differed from reference values by less than 1 SD were scored as 0. Values that were ±1 SD with respect to the reference value were given a frailty value of 0.25; values that differed by ±2 SD scored 0.5, those that differed by ±3 SD scored 0.75 and values that were >3 SD above or below the mean received the maximal frailty value of 1.

The sum retrieved from the values assigned to the 31 items on the checklist was then divided by 31 to yield a FI score between 0 and 1 for each animal.

The measurement of physical frailty in mice was performed following the same procedure described to translate the physical frailty screening performed in humans [1] to mice [11, 20, 31]. Performance testing was performed in both old and adult mice at the 3rd month from the start of the study (before the treatment period) and at the 7th month (after the treatment period). In order to ensure testing reliability, we adapted the mice to the tests for at least 2 months before the start of the study and performed multiple measurements for each criterion of the frailty assessment. The results from the multiple measurement were combined in a unique score for each criterion. The same testers performed all the measurements of frailty. All measurements performed to define the Physical Frailty phenotype are schematically described in Supplementary Table 3.

Our frailty phenotype included the following physical components:

1. Shrinking (weight loss). Shrinking was assessed by recording the current body weight and changes of body weight (these last measurements were obtained by comparing the current weight with the one measured in the previous 1 and 2 months).

2. Weakness. This criterion was assessed by measuring forelimb grip strength with 3 different tests: grip strength meter (Ugo Basile, Varese, Italy) measurement [32], dynamometer force measurement and increasing weights lift test [33].

3. Endurance. This criterion was measured by treadmill distance (program: starting at 5 rpm for 2 min and increasing speed from 5 to 50 m/s in 2700 s), mean time to fall at rotarod test (program: starting at 5 m/s for 2 min and increasing speed from 5 to 40 rpm in 300 s) and max weight reached at the increasing weights lift test. This test includes an endurance component due to the continuous increasing of the weight to be lifted by the mouse [33].

4. Slowness. We assessed this criterion by analyzing the distribution of the time spent by the mouse in different speed intervals in an Open Field test (whole test duration 5 min). The speed intervals considered where: I1 (0-1 cm/s), I2 (1-5 cm/s), I3 (5-10 cm/s), I4 (10-15 cm/s), I5 (15-20 cm/s), I6 (20-25 cm/s), I7 (25-30 cm/s), I8 (30-35 cm/s), I9 (35-40 cm/s), I10 (40-90 cm/s). We recorded the highest speed interval that the mouse run for at least 3 s and assigned as value of the test the mean speed of the interval (e.g. 12.5 for I4 and 37.5 cm/s for I9). The threshold of 3 s was established based on association with mortality data obtained from other cohort of mice (data not shown). Locomotor activity was conducted by a 5-min open field test on a white wood-chamber (72×72×30 cm) surmounted by a Xiaomi Yi Camera 16MP 1080 P 60FPS (YI Technology) controlled WI-Fi by a Smartphone. Videos were collected in a microSD disk and the tracking was performed offline with Biobserve Viewer3 (Biobserve GmbH, Germany) as previously described [12]. An additional

measurement for slowness was obtained by recording the max speed recorded at rotarod test. Furthermore, we assessed slowness by also including the measurement of the mean stride length of the mice following a previously established protocol [34]. Indeed, there is a strong rationale in support of the relationship between walking speed and stride length, especially in older individuals [35].

5. Activity. Activity was recorded automatically by Biobserve Viewer3 (Biobserve GmbH, Germany) as the % the mice walked or run (speed above 0.45 cm/s) in a 5-min open field test. We additionally recorded the total distance run by the mouse in the same test.

All variables obtained by the measurements described above were standardized (transformed into Z-scores) and the variables assigned to the same criterion

were averaged to create a composite Z-score. Following the percentiles used by Fried et al. in humans [1] and by others in mice [20], mice that fell in the bottom 20% of our old cohort for the composite Z-score computed for each criterion (Shrinking, Weakness, Endurance, Slowness and Activity), were considered positive for frailty for that given criterion. Mice with three or more positive frailty criteria were identified as frail.

Statistical analysis

Human study

For all the analyses, we re-codified the classification at *BPIFB4* locus taking into account the haplotypic phase, as previously reported [23]. Since the LAV is represented by the minor allele of the two SNPs in haplotypic phase, the association with frailty trait was tested assuming a recessive model for this allele (i.e., LAV homozygotes – defined as rs2070325 = G and rs11699009 = T on both chromosomes – vs. LAV heterozygotes – defined as rs2070325 = G and rs11699009 = T on one chromosome – plus remaining haplotype carriers pooled). For the remaining two haplotypes with frequency >1% (i.e., RV and WT), dominant and recessive genetic models were assumed respectively (i.e., for RV, RV haplotype carriers – defined as rs2070325 = A and rs11699009 = T on at least one chromosome – vs. non-carriers; and for WT, WT homozygotes – defined as rs2070325 = A and rs11699009 = C on both chromosomes – vs. WT heterozygotes – defined as rs2070325 = A and rs11699009 = C on one chromosome – plus remaining haplotype alleles pooled, respectively).

Haplotype frequencies and phases were estimated from the observed genotypes. Fisher's Exact test were used for the comparison of frequencies of analyzed haplotypes. In order to evaluate if the detected effects of the analyzed polymorphisms on frailty status might finally result in differential patterns of survival of the different relevant genotypes, the survival after 11 years from the baseline visit was estimated. Kaplan–Meier survival curves were estimated for carriers vs non carriers of the

relevant haplotype; in order to evaluate their predictive value with respect to mortality risk, the obtained survival curves were then compared by log-rank test. Hazard Ratios (HR) and 95% Confidence Intervals (95% CI) were estimated by using Cox proportional hazard models taking also into account possible confounder variables (age, gender, frailty status). Subjects alive after the follow-up time were considered as censored, and this time was used as the censoring date in the survival analyses. All the analyses were performed in R environment [36] and SPSS 25.0 (SPSS Inc., Chicago, IL). A significance level (α) of 0.05 was chosen in all the tests.

Animal study

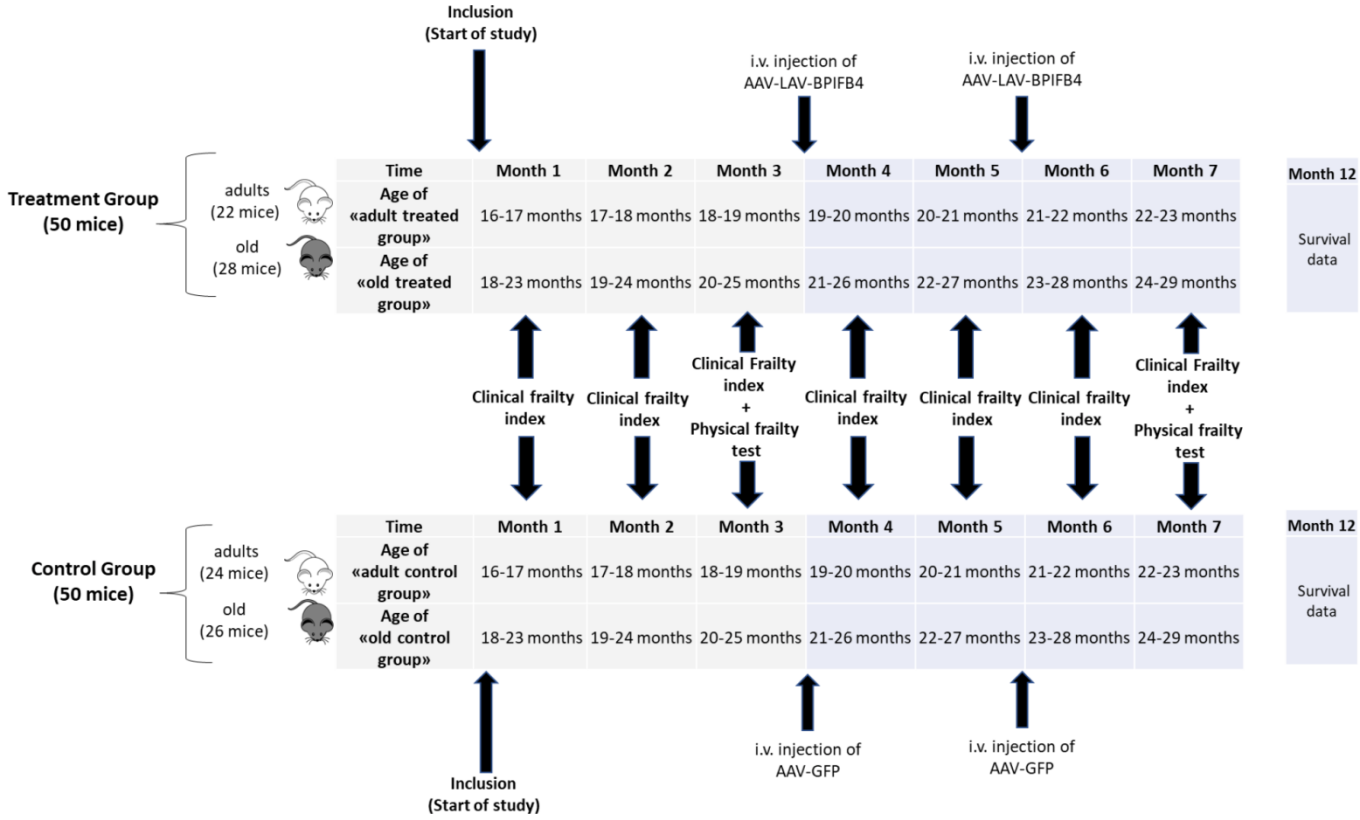
Generalized linear mixed model analysis (SPSS 25.0) was used to take into account the longitudinal design of the study in mice. The identifier of each mouse, age group, gender, age of mouse at inclusion and time was indicated in the model. The linear model was developed assuming normal distribution with identity link function for data of the Clinical Frailty Index. The Satterthwaite approximation and robust estimator were used to take into account unbalanced data and violation of the assumptions. Fisher exact test was used to compare the prevalence of the physical frailty phenotype between control and treated animals. Differential patterns of survival due to the treatment were estimated by Cox-regression taking also into account possible confounder variables (age, age group and gender).

References

1. Fried LP, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, Seeman T, Tracy R, Kop WJ, Burke G, McBurnie MA, and Cardiovascular Health Study Collaborative Research Group. Frailty in older adults: evidence for a phenotype. J Gerontol A Biol Sci Med Sci. 2001; 56:M146–56.
2. Buckinx F, Rolland Y, Reginster JY, Ricour C, Petermans J, Bruyère O. Burden of frailty in the elderly population: perspectives for a public health challenge. Arch Public Health. 2015; 73:19.
3. Song X, Mitnitski A, Rockwood K. Prevalence and 10-year outcomes of frailty in older adults in relation to deficit accumulation. J Am Geriatr Soc. 2010; 58:681–87.
4. Villa F, Carrizzo A, Ferrario A, Maciag A, Cattaneo M, Spinelli CC, Montella F, Damato A, Ciaglia E, Puca AA. A Model of Evolutionary Selection: The Cardiovascular Protective Function of the Longevity Associated Variant of BPIFB4. Int J Mol Sci. 2018; 19:3229.
5. Malovini A, Illario M, Iaccarino G, Villa F, Ferrario A, Roncarati R, Anselmi CV, Novelli V, Cipolletta E, Leggiero E, Orro A, Rusciano MR, Milanesi L, et al. Association study on long-living individuals from Southern Italy identifies rs10491334 in the CAMKIV gene that regulates survival proteins. Rejuvenation Res. 2011; 14:283–91.
6. Santulli G, Cipolletta E, Sorriento D, Del Giudice C, Anastasio A, Monaco S, Maione AS, Condorelli G, Puca A, Trimarco B, Illario M, Iaccarino G. CaMK4 Gene Deletion Induces Hypertension. J Am Heart Assoc. 2012; 1:e001081.
7. Villa F, Malovini A, Carrizzo A, Spinelli CC, Ferrario A, Maciąg A, Madonna M, Bellazzi R, Milanesi L, Vecchione C, Puca AA. Serum BPIFB4 levels classify health status in long-living individuals. Immun Ageing. 2015; 12:27.
8. Spinetti G, Sangalli E, Specchia C, Villa F, Spinelli C, Pipolo R, Carrizzo A, Greco S, Voellenkle C, Vecchione C, Madeddu P, Martelli F, Puca AA. The expression of the BPIFB4 and CXCR4 associates with sustained health in long-living individuals from Cilento-Italy. Aging (Albany NY). 2017; 9:370–80.
9. Villa F, Carrizzo A, Spinelli CC, Ferrario A, Malovini A, Maciąg A, Damato A, Auricchio A, Spinetti G, Sangalli E, Dang Z, Madonna M, Ambrosio M, et al. Genetic analysis reveals a longevity-associated protein modulating endothelial function and angiogenesis. Circ Res. 2015; 117:333–45.
10. Montesanto A, Lagani V, Martino C, Dato S, De Rango F, Berardelli M, Corsonello A, Mazzei B, Mari V, Lattanzio F, Conforti D, Passarino G. A novel, population-specific approach to define frailty. Age (Dordr). 2010; 32:385–95.
11. Liu H, Graber TG, Ferguson-Stegall L, Thompson LV. Clinically relevant frailty index for mice. J Gerontol A Biol Sci Med Sci. 2014; 69:1485–91.
12. Malavolta M, Basso A, Giacconi R, Orlando F, Pierpaoli E, Cardelli M, Leoni F, Chierichetti S, Bray D, Benlhassan K, Provinciali M. Recovery from mild Escherichia coli O157:H7 infection in young and aged C57BL/6 mice with intact flora estimated by fecal shedding, locomotor activity and grip strength. Comp Immunol Microbiol Infect Dis. 2019; 63:1–9.
13. Basso A, Del Bello G, Piacenza F, Giacconi R, Costarelli L, Malavolta M. Circadian rhythms of body temperature and locomotor activity in aging BALB/c mice: early and late life span predictors. Biogerontology. 2016; 17:703–14.

14. Whitehead JC, Hildebrand BA, Sun M, Rockwood MR, Rose RA, Rockwood K, Howlett SE. A clinical frailty index in aging mice: comparisons with frailty index data in humans. J Gerontol A Biol Sci Med Sci. 2014; 69:621–32.
15. Fried LP, Ferrucci L, Darer J, Williamson JD, Anderson G. Untangling the concepts of disability, frailty, and comorbidity: implications for improved targeting and care. J Gerontol A Biol Sci Med Sci. 2004; 59:255–63.
16. Rockwood K, Song X, MacKnight C, Bergman H, Hogan DB, McDowell I, Mitnitski A. A global clinical measure of fitness and frailty in elderly people. CMAJ. 2005; 173:489–95.
17. Ritt M, Schwarz C, Kronawitter V, Delinic A, Bollheimer LC, Gassmann KG, Sieber CC. 's frailty phenotype as predictors of mortality and other clinical outcomes in older patients who were admitted to a geriatric ward. J Nutr Health Aging. 2015; 19:1043–48.
18. Feridooni HA, Sun MH, Rockwood K, Howlett SE. Reliability of a Frailty Index Based on the Clinical Assessment of Health Deficits in Male C57BL/6J Mice. J Gerontol A Biol Sci Med Sci. 2015; 70:686–93.
19. Dato S, Montesanto A, Lagani V, Jeune B, Christensen K, Passarino G. Frailty phenotypes in the elderly based on cluster analysis: a longitudinal study of two Danish cohorts. Evidence for a genetic influence on frailty. Age (Dordr). 2012; 34:571–82.
20. Baumann CW, Kwak D, Thompson LV. Assessing onset, prevalence and survival in mice using a f railty phenotype. Aging (Albany NY). 2018; 10:4042–53.
21. Alonso-Bouzón C, Carcaillon L, García-García FJ, Amor-Andrés MS, El Assar M, Rodríguez-Mañas L. Association between endothelial dysfunction and frailty: the Toledo Study for Healthy Aging. Age (Dordr). 2014; 36:495–505.
22. Mansur HN, Lovisi JC, Colugnati FA, Raposo NR, Fernandes NM, Bastos MG. Association of frailty with endothelial dysfunction and its possible impact on negative outcomes in Brazilian predialysis patients with chronic kidney disease. BMC Nephrol. 2015; 16:157.
23. Vecchione C, Villa F, Carrizzo A, Spinelli CC, Damato A, Ambrosio M, Ferrario A, Madonna M, Uccellatore A, Lupini S, Maciag A, Ryskalin L, Milanesi L, et al. A rare genetic variant of BPIFB4 predisposes to high blood pressure via impairment of nitric oxide signaling. Sci Rep. 2017; 7:9706.
24. Veronese N, Sigeirsdottir K, Eiriksdottir G, Marques EA, Chalhoub D, Phillips CL, Launer LJ, Maggi S, Gudnason V, Harris TB. Frailty and Risk of Cardiovascular Diseases in Older Persons: The Age, Gene/Environment Susceptibility-Reykjavik Study. Rejuvenation Res. 2017; 20:517–24.
25. Collerton J, Martin-Ruiz C, Davies K, Hilkens CM, Isaacs J, Kolenda C, Parker C, Dunn M, Catt M, Jagger C, von Zglinicki T, Kirkwood TB. Frailty and the role of inflammation, immunosenescence and cellular ageing in the very old: cross-sectional findings from the Newcastle 85+ Study. Mech Ageing Dev. 2012; 133:456–66.
26. Soysal P, Stubbs B, Lucato P, Luchini C, Solmi M, Peluso R, Sergi G, Isik AT, Manzato E, Maggi S, Maggio M, Prina AM, Cosco TD, et al. Inflammation and frailty in the elderly: A systematic review and meta-analysis. Ageing Res Rev. 2016; 31:1–8.
27. Puca AA, Carrizzo A, Spinelli C, Damato A, Ambrosio M, Villa F, Ferrario A, Maciag A, Fornai F, Lenzi P, Valenti V, di Nonno F, Accarino G, et al. Single systemic transfer of a human gene associated with exceptional longevity halts the progression of atherosclerosis and inflammation in ApoE knockout mice through a CXCR4-mediated mechanism. Eur Heart J. 2019; [Epub ahead of print].

28. Ciaglia E, Montella F, Maciag A, et al. Longevity-Associated Variant of BPIFB4 Mitigates Monocyte-Mediated Acquired Immune Response. J Gerontol A Biol Sci Med Sci. 2019. [Epub ahead of print].
29. Garcia-Valles R, Gomez-Cabrera MC, Rodriguez-Mañas L, Garcia-Garcia FJ, Diaz A, Noguera I, Olaso-Gonzalez G, Viña J. Life-long spontaneous exercise does not prolong lifespan but improves health span in mice. Longev Healthspan. 2013; 2:14.
30. Mitchell SJ, Bernier M, Aon MA, Cortassa S, Kim EY, Fang EF, Palacios HH, Ali A, Navas-Enamorado I, Di Francesco A, Kaiser TA, Waltz TB, Zhang N, et al. Nicotinamide Improves Aspects of Healthspan, but Not Lifespan, in Mice. Cell Metab. 2018; 27:667–676.e4.
31. Kane AE, Huizer-Pajkos A, Mach J, et al. A Comparison of Two Mouse Frailty Assessment Tools. J Gerontol A Biol Sci Med Sci. 2017; 72:904–909.
32. Castro B, Kuang S. Evaluation of Muscle Performance in Mice by Treadmill Exhaustion Test and Whole-limb Grip Strength Assay. Bio Protoc. 2017; 7:e2237. 33. Deacon RM. Measuring the strength of mice. J Vis Exp. 2013.
34. Fernagut PO, Diguet E, Labattu B, Tison F. A simple method to measure stride length as an index of nigrostriatal dysfunction in mice. J Neurosci Methods. 2002; 113:123–30.
35. Morio Y, Izawa KP, Omori Y, Katata H, Ishiyama D, Koyama S, Yamano Y. The Relationship between Walking Speed and Step Length in Older Aged Patients. Diseases. 2019; 7:17.
36. Team RD. A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria: ISBN; 2009.



Supplementary Figure 1. Schematic of the study design adopted*. ***The age of the mice refers to the range of age at the beginning of the indicated month from the inclusion.

Supplementary Table 1. Prevalence of physical frailty (Fried's phenotype) in treated and control mice at the 3rd month (before treatment) and at the 7th month (after treatment) from the inclusion in the study*.

Age of mice at the beginning of the study	Status	Month 3 (Before treatment)		Month 7 (Post treatment)	
		Control	LAV-*BPIFB4*	Control	LAV-*BPIFB4*
Adult mice	Frail	2 (8.3%)	1 (4.5%)	2 (9.5%)	1 (4.8%)
	Non-frail	22 (91.7%)	21 (95.5%)	19 (90.5%)	20 (95.2%)
Old mice	Frail	4 (15.4%)	3 (10.7%)	8 (44.4%)	5 (20%)
	Non-frail	22 (84.6%)	25 (89.3%)	10 (55.6%)	20 (80%)

*Data are reported as number of mice (%). At month 3: p = 0.741 by Fisher's exact test; At month 7: p = 0.170 by Fisher's exact test; The reduced number of mice at month 7 is due to death events occurring during the study.

Supplementary Table 2. Number of mice (%) with frailty scores of 0, 0.5 or 1.0 for each parameter used to develop the frailty index.

		Months from the beginning of the study													
		3 months (Before treatment)								7 months (Post treatment)					
		Adult Controls		Adult LAV		Old Controls		Old LAV		Adult Controls		Adult LAV		Old Controls		Old LAV	
Item	**Score**	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Alopecia	**0**	17	74%	17	77%	13	52%	17	61%	14	67%	11	58%	7	44%	12	67%
	0.5	5	22%	4	18%	9	36%	11	39%	7	33%	7	37%	6	38%	5	28%

	1	1	4%	1	5%	3	12%	0	0%	0	0%	1	5%	3	19%	1	6%
Loss of fur color	**0**	14	61%	11	50%	14	56%	17	61%	7	33%	5	26%	0	0%	4	22%
	0.5	9	39%	11	50%	11	44%	10	36%	11	52%	10	53%	11	69%	11	61%
	1	0	0%	0	0%	0	0%	1	4%	3	14%	4	21%	5	31%	3	17%
Dermatitis	**0**	19	83%	18	82%	25	100%	27	96%	20	95%	16	84%	16	100%	17	94%
	0.5	4	17%	3	14%	0	0%	0	0%	0	0%	1	5%	0	0%	0	0%
	1	0	0%	1	5%	0	0%	1	4%	1	5%	2	11%	0	0%	1	6%
Loss of whiskers	**0**	11	48%	8	36%	8	32%	11	39%	7	33%	5	26%	5	31%	7	39%
	0.5	1	4%	2	9%	6	24%	7	25%	1	5%	2	11%	5	31%	3	17%
	1	11	48%	12	55%	11	44%	10	36%	13	62%	12	63%	6	38%	8	44%
Coat condition	**0**	19	83%	17	77%	12	48%	18	64%	6	29%	8	42%	4	25%	6	33%
	0.5	3	13%	5	23%	12	48%	10	36%	13	62%	5*	26%	9	56%	11	61%
	1	1	4%	0	0%	1	4%	0	0%	2	10%	6	32%	3	19%	1	6%
Tumours/Lipomas	**0**	22	96%	21	96%	21	84%	27	96%	20	95%	19	100%	13	81%	15	83%
	0.5	0	0%	1	5%	3	12%	0	0%	1	5%	0	0%	1	6%	0	0%
	1	1	4%	0	0%	1	4%	1	4%	0	0%	0	0%	2	13%	3	17%
Distended abdomen	**0**	21	91%	22	100%	25	100%	26	93%	17	81%	12	63%	9	56%	13	72%
	0.5	2	9%	0	0%	0	0%	2	7%	3	14%	7	37%	6	38%	4	22%
	1	0	0%	0	0%	0	0%	0	0%	1	5%	0	0%	1	6%	1	6%
Kyphosis	**0**	23	100%	20	91%	16	64%	23	82%	8	38%	6	32%	1	6%	3	17%
	0.5	0	0%	2	9%	8	32%	5	18%	13	62%	12	63%	8	50%	12	67%
	1	0	0%	0	0%	1	4%	0	0%	0	0%	1	5%	7	44%	3	17%
Tail stiffening	**0**	23	100%	22	100%	25	100%	27	96%	15	71%	16	84%	11	69%	11	61%
	0.5	0	0%	0	0%	0	0%	1	4%	6	29%	3	16%	5	31%	7	39%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Gait disorders	**0**	16	70%	14	64%	13	52%	18	64%	11	52%	13	68%	3	19%	11*	61%
	0.5	7	30%	8	36%	12	48%	10	36%	9	43%	5	26%	10	63%	7	39%
	1	0	0%	0	0%	0	0%	0	0%	1	5%	1	5%	3	19%	0	0%
Tremor	**0**	23	100%	22	100%	25	100%	28	100%	20	95%	17	90%	10	63%	17*	94%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	2	11%	2	13%	1	6%
	1	0	0%	0	0%	0	0%	0	0%	1	5%	0	0%	4	25%	0	0%
Forelimb grip strength	**0**	22	96%	20	91%	24	96%	24	86%	20	95%	18	95%	11	69%	17*	94%
	0.5	1	4%	2	9%	1	4%	4	14%	1	5%	1	5%	2	13%	1	6%

	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	3	19%	0	0%
Body condition	**0**	16	70%	16	73%	19	76%	23	82%	14	67%	14	74%	9	56%	13	72%
	0.5	7	30%	4	18%	5	20%	5	18%	6	29%	4	21%	6	38%	5	28%
	1	0	0%	2	9%	1	4%	0	0%	1	5%	1	5%	1	6%	0	0%
Vestibular disturbance	**0**	23	100%	22	100%	25	100%	28	100%	21	100%	18	95%	10	63%	17*	94%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	1	5%	2	13%	1	6%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	4	25%	0	0%
Hearing loss	**0**	11	48%	8	36%	11	44%	10	36%	4	19%	3	16%	0	0%	3	17%
	0.5	9	39%	9	41%	6	24%	8	29%	11	52%	10	53%	4	25%	7	39%
	1	3	13%	5	23%	8	32%	10	36%	6	29%	6	32%	12	75%	8	44%
Cataracts	**0**	23	100%	22	100%	23	92%	28	100%	21	100%	18	95%	16	100%	18	100%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	1	5%	0	0%	0	0%
	1	0	0%	0	0%	2	8%	0	0%	0	0%	0	0%	0	0%	0	0%
Corneal opacity	**0**	22	96%	21	96%	25	100%	27	96%	20	95%	19	100%	15	94%	17	94%
	0.5	1	4%	1	5%	0	0%	0	0%	1	5%	0	0%	1	6%	0	0%
	1	0	0%	0	0%	0	0%	1	4%	0	0%	0	0%	0	0%	1	6%
Eye discharge/swelling	**0**	21	91%	21	96%	22	88%	24	86%	14	67%	12	63%	9	56%	9	50%
	0.5	2	9%	1	5%	3	12%	4	14%	6	29%	7	37%	4	25%	8	44%
	1	0	0%	0	0%	0	0%	0	0%	1	5%	0	0%	3	19%	1	6%
Microphtalmia	**0**	23	100%	22	100%	25	100%	28	100%	17	81%	14	74%	12	75%	15	83%
	0.5	0	0%	0	0%	0	0%	0	0%	3	14%	5	26%	2	13%	3	17%
	1	0	0%	0	0%	0	0%	0	0%	1	5%	0	0%	2	13%	0	0%
Vision loss	**0**	1	4%	1	5%	1	4%	0	0%	1	5%	0	0%	0	0%	0	0%
	0.5	7	30%	10	46%	5	20%	9	32%	5	24%	5	26%	1	6%	3	17%
	1	15	65%	11	50%	19	76%	19	68%	15	71%	14	74%	15	94%	15	83%
Manace reflex	**0**	22	96%	18	82%	24	96%	27	96%	2	10%	1	5%	1	6%	1	6%
	0.5	1	4%	4	18%	1	4%	1	4%	9	43%	8	42%	2	13%	5	28%
	1	0	0%	0	0%	0	0%	0	0%	10	48%	10	53%	13	81%	12	67%
Nasal discharge	**0**	23	100%	22	100%	25	100%	28	100%	21	100%	19	100%	15	94%	17	94%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	6%	1	6%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Malocclusion	**0**	22	96%	22	100%	25	100%	28	100%	20	95%	18	95%	14	88%	18	100%
	0.5	1	4%	0	0%	0	0%	0	0%	1	5%	1	5%	2	13%	0	0%

	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Rectal prolapse	**0**	23	100%	22	100%	25	100%	27	96%	21	100%	19	100%	16	100%	17	94%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	6%
	1	0	0%	0	0%	0	0%	1	4%	0	0%	0	0%	0	0%	0	0%
Vaginal/uterine/penile prolapse	**0**	23	100%	22	100%	25	100%	28	100%	17	81%	18	95%	16	100%	16	89%
	0.5	0	0%	0	0%	0	0%	0	0%	2	10%	1	5%	0	0%	2	11%
	1	0	0%	0	0%	0	0%	0	0%	2	10%	0	0%	0	0%	0	0%
Diarrhea	**0**	23	100%	22	100%	25	100%	28	100%	21	100%	19	100%	15	94%	18	100%
	0.5	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	6%	0	0%
Breathing rate/depth	**0**	22	96%	22	100%	25	100%	28	100%	16	76%	17	90%	7	44%	13	72%
	0.5	0	0%	0	0%	0	0%	0	0%	4	19%	2	11%	8	50%	4	22%
	1	1	4%	0	0%	0	0%	0	0%	1	5%	0	0%	1	6%	1	6%
Mouse grimace scale	**0**	23	100%	21	96%	25	100%	28	100%	18	86%	16	84%	9	56%	13	72%
	0.5	0	0%	1	5%	0	0%	0	0%	3	14%	3	16%	2	13%	4	22%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	5	31%	1*	6%
Pilorection	**0**	23	100%	21	96%	19	76%	20	71%	11	52%	9	47%	0	0%	5	28%
	0.5	0	0%	1	5%	6	24%	7	25%	8	38%	6	32%	9	56%	11*	61%
	1	0	0%	0	0%	0	0%	1	4%	2	10%	4	21%	7	44%	2	11%
Weight score	**0**	12	52%	16	73%	17	68%	17	61%	9	43%	9	47%	8	50%	9	50%
	0.25	3	13%	1	5%	3	12%	1	4%	2	10%	4	21%	0	0%	4	22%
	0.5	7	30%	5	23%	3	12%	8	29%	7	33%	6	32%	5	31%	3	17%
	0.75	1	4%	0	0%	2	8%	2	7%	3	14%	0	0%	3	19%	2	11%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Temperature score	**0**	19	83%	18	82%	19	76%	25	89%	14	67%	18*	95%	9	56%	13	72%
	0.25	2	9%	4	18%	6	24%	2	7%	6	29%	1	5%	4	25%	4	22%
	0.5	2	9%	0	0%	0	0%	1	4%	1	5%	0	0%	1	6%	1	6%
	0.75	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	6%	0	0%
	1	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%

Supplementary Table 3. Measurement performed to define Physical Frailty phenotype*.

Criterion	Shrinking	Weakness	Endurance	Slowness	Activity
Measurement 1	Current weight	Grip strength meter	Treadmill distance	Highest speed interval that the mouse traveled for at least 3 s in an open field test (5 min)	% the mice walked or run in a 5-min open field test
Measurement 2	Weight loss in 1 month	Dynamometer force	Mean time at Rotarod test	Mean stride length	Total distance run by the mouse in a 5-min open field test
Measurement 3	Weight loss in 2 month	Increasing weights lift test	Increasing weights lift test	Max speed at rotarod test	-

*For each criterion a composite Z-score was derived as the mean of the Z-scores from each measurement. Mice that fell in the bottom 20% of our cohort for the composite score computed for each criterion (Shrinking, Weakness, Endurance, Slowness and Activity), were assigned one point. The mice were considered as frail when they reached 3 or more points on a maximum of 5.

CHAPTER 6

Expression patterns of muscle-specific miR-133b and miR-206 correlate with nutritional status and affect sarcopenia

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1. Introduction

The wide-ranging variation in the rate and quality of aging results from the intertwined interactions among a variety of genetic/epigenetic and environmental/lifestyle factors that impinge lifelong on our body. Nutrition is seen as one of the most important modifiable lifestyle factor affecting the whole aging process, with evidences increasingly indicating that nutrition is a major risk factor for the onset of chronic conditions [1,2]. Many changes accompanying aging, such as anorexia of aging, body composition changes, worsening of oral health and decline of sensory functions as well as pathological and socio-environmental factors, can promote a poor nutritional status due to inadequate nutritional intake [3]. Malnutrition represents a common problem in older persons, with data showing that up to 22% of older adults are malnourished and over 45% are at risk of malnutrition [4]. The consequences of malnutrition are diverse, severe and long-lasting. People with a poor nutritional status experience an accelerated transition from vulnerability to frailty and dependence and also are at increased risk of mortality [5,6,7].

The skeletal muscle is an adaptive tissue involved in the global metabolic homeostasis regulation. It is a key site for glucose uptake and storage and the largest reservoir of proteins and free amino acids in the body that plays a crucial role in the global metabolic homeostasis via inter-organ crosstalk [8]. As the largest metabolic organ in the body, skeletal muscle is strongly influenced by the nutritional status. Indeed, malnutrition, together with factors related to age such as chronic inflammation, oxidative stress, and hormonal changes, is a key contributor to development of sarcopenia, the progressive and generalized loss of muscle mass and strength that accompanies aging and the leading cause of disability, morbidity, and mortality in older adults [9,10,11,12]. According to the current estimates, 5–10% of elderly people aged 60–70 years and 11–50% of those over the age of 80 are facing with this disability [13]. Thus, these statistics prompt this issue as a serious public health concern.

The maintenance of muscle homeostasis is finely regulated by the orchestrate action of muscle-specific transcription factors and epigenetic regulators. These include DNA methylation, histone modification, as well as the non-coding microRNAs (miRNAs) [14]. MiRNAs are small molecules, approximately 21-22 nucleotides in length, able to regulate the expression of their targets by binding to 5′UTR, coding regions or 3′UTR of mRNAs, causing translational inhibition or mRNA degradation [15]. Because of their versatility (the same miRNAs can target a large number of mRNAs, and the same mRNA can be targeted by many miRNAs), miRNAs exert extensive regulatory control over various biological processes, greatly influencing both physiological and pathological processes [16]. Recently, several miRNAs have been found up- or down-regulated in

skeletal muscle during aging (reviewed in [17]), suggesting that this differential expression may underlie the reduced age-related muscle functionality [18,19,20,21]. In particular, a pivotal role in almost all aspects of skeletal muscle developments is presently assigned to a group of specific skeletal muscle miRNAs, designated myomiRs. This group comprises miRNA-1, miRNA-133a, miRNA-133b, miRNA-206, miRNA-208b, and miRNA-499, whose characteristic features are shown in Supplementary Material, Figure S1. They play a crucial role in the context of muscle physiology by targeting genes involved in signaling pathways that regulate muscle skeletal development and growth, differentiation and regeneration [22,23,24,25,26]. An interesting clue is that many of them can regulate, or be regulated, by components of the IGF-1/Akt/mTOR signaling pathway, known to regulate skeletal muscle protein synthesis (MPS) and muscle protein breakdown (MPB) [27], two processes that are highly responsive to anabolic stimuli, such as physical activity and food intake. Interestingly, myomiRs expression has been shown to be modulated by exercise, although the directionality of these changes appears to be sensitive to exercise type [28,29], and by the combined ingestion of essential amino acid [30,31], proteins [32] and carbohydrates [31]. This is particularly intriguing, considering that the imbalance between MPS and MPB, associated with impaired rate of muscle anabolic responses to exercise and nutrients (in particular protein intake), termed “anabolic resistance”, may underpin the progression of sarcopenia [33]. Moreover, the study by Drummond and colleagues [30] also examined myomiR expression patterns in muscle in relation to aging, detecting no differences in basal expression of mature functional myomiRs between elderly and younger men. Yet, the expression of myomiRs in response to resistance exercise and ingestion of essential amino acids was different between elderly and young. However, contrasting results were found by Nielsen and colleagues [34], who reported higher expression of miR-1 and 133a/b in older men compared to young men.

As far as we know, there are no studies evaluating circulating myomiRs levels in sarcopenic subjects and the possible relationship with nutrition. Thus, the main objective of this study was 2-fold: firstly, to examine whether expression levels of myomiRs transcripts in old individuals are associated with sarcopenia; and secondly, to explore whether the associations are influenced by nutritional status. This study, by identifying those myomiRs changing their expression in sarcopenic subjects, may provide new useful biomarkers of sarcopenia. Furthermore, the potential relationship with nutritional status could provide insights in the molecular pathways underpinning sarcopenia and help to develop therapeutic targets or set up lifestyle interventions to prevent or delay the onset of the age-related muscle decline.

2. Materials and methods

Participants

Participants were recruited from elderly nursing homes located in the province of Crotone and Cosenza in the Calabria region (southern Italy), as part of a study carried out for monitoring the quality of aging in the whole region. Subjects were eligible to participate in the study if they were older than 65 years of age and of Calabrian ancestry. We excluded patients with cardiac involvement and severe neuropsychiatric illness that caused patients to be unable to understand and perform instructions and to provide written informed consent. In total, 218 subjects were enrolled, 79 were males and 139 females with a mean age of 81.6 (± 7.10) years. At recruitment, eligible and consenting participants were subjected to a multidimensional geriatric assessment including information on demographics (age, sex, education), cognitive status, functional abilities, and physical health, obtained through a structured questionnaire administered during an interview with a trained operator. Peripheral blood sample was collected from each participant for clinical and laboratory examinations.

Ethics Statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local Ethical Committee (Comitato Etico Regione Calabria-Sezione Area Nord) on 2017-10-31 (code n. 25/2017).

Assessment of muscle mass

Muscle mass was measured by Bioelectrical impedance analysis (BIA) using a Quantum/S Bioelectrical Body Composition Analyzer (AkernSrl, Florence, Italy). Whole-body BIA measurements were taken between the right wrist and ankle with subject in a supine position. Muscle mass was calculated using the BIA equation of Janssen and colleagues [35]: Skeletal muscle mass (kg) = ([height^2/BIA resistance × 0.401] + [gender × 3.825] - [age × 0.071]) + 5.102, where height is measured in centimeters; bioelectrical impedance analyses resistance is measured in ohms; for gender, men = 1 and women = 0; age is measured in years. Absolute muscle mass was converted to skeletal muscle index (SMI) by dividing the value by the square of the height in meters (kg/m^2).

Measurement of muscle strength

Muscle strength was assessed as hand grip strength (HGS) using a handheld dynamometer (SMEDLEY's dynamometer TTM) while the subject was sitting with the arm close to his/her body.

The test was repeated three times with the stronger hand and the maximum of these values was considered.

Assessment of muscle performance

Gait speed was measured using the 4-meter walking test. Patients were asked to walk straight for 4 meters at their usual speed for measurement of 4-meter walk time. Timing began when subjects initiated foot movement and stopped when 1 foot contacted the ground after completely crossing the 4 meters' mark. Gait speed (m/sec) was calculated by dividing the distance covered (4 meters (m)) by the 4-meter walk time (sec). The best time of 2 attempts was recorded.

Evaluation of Disability

The management of Activities of Daily Living or ADL (bathing, dressing, toileting, transfer from bed to chair, and feeding) was assessed using a modification of the Katz Index of ADL [36]. The assessment was based on what the subject was able to do at the time of the visit. The score is given counting the number of activities in which the participant is dependent or independent at the time of the visit. For the analyses, ADL scores were dichotomized as one if the subject was not independent in all five items and zero otherwise.

Nutritional assessment

We used the Mini Nutritional Assessment (MNA) to assess the nutritional state of the participants [37]. The short form of the MNA (MNA-SF) includes 6 queries regarding food intake, weight loss, mobility, psychological stress, or acute disease, the presence of dementia or depression, and body mass index (BMI). For this screening tool, the maximum score is equal to 14. A score ≥12 indicates that the subject has an acceptable nutritional status, whereas a score < 12 indicates risk of malnutrition; in this last case it was necessary to complete the MNA Long Form (MNA-LF). The MNA-LF consists of 18 items from 4 sections: global evaluation, anthropometric assessment, dietetic assessment (including number of full meals, fruit/vegetables, and water consumption), and self-assessment. The total score ranges from 0 to 30. Individuals were considered malnourished if they score <17, at risk for malnutrition if they score between 17 and 23.5, and well-nourished if their scores are ≥24.

Anthropometrics and biochemical markers of nutritional status reported in Table 1 were measured using standard laboratory procedures in all the subjects.

Diagnosis of Sarcopenia

Sarcopenia was diagnosed by measuring muscle strength, muscle mass, and physical performance according to the revised criteria suggested by the European Working Group on Sarcopenia in Older People (EWGSOP2) [38]. Sarcopenia was defined as low muscle strength (HGS < 27 kg in males; < 16 kg in females) associated with either low skeletal muscle mass index (SMI; < 8.50 kg/m2 in males; < 5.75 kg/m2 in females) or low gait speed (< 0.8 m/s).

Blood plasma collection

Blood plasma samples were prepared as follows. Venous blood samples were drawn after 12-hours overnight fast and processed within 2 hours from collection. Plasma for miRNAs analysis was separated by centrifugation at 1,800g for 10 minutes at room temperature, collected in RNAse-free tubes and further centrifuged at 1,200g for 20 minutes at 10°C to completely remove contaminant cells. Finally, plasma samples were divided in aliquots to avoid freeze–thaw cycles and finally stored at −80°C up to the RNA extraction.

RNA Extraction and MiRNA Quantification

miRNAs were isolated from 200 µl of plasma using miRNeasy® Serum/Plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 5 volumes of QIAzol Lysis Reagent were added to each sample. After 5 min incubation at room temperature, 200 µl of chloroform were added together with 3.5 µl of Arabidopsis thaliana miR-159a (assay ID 000338) as a spike-in control and the mixture was incubated for 3 minutes. The lysate was separated into aqueous and organic phases by centrifugation for 15 minutes at 12,000 x g. 1.5 volumes of 100% ethanol were added to the aqueous phase and the solution was passed through the RNeasy MinElute spin column in order to make the small RNAs tie to the membrane. Using appropriate washing buffers, phenol and other contaminants were expelled. Finally, RNAs was washed with 80% ethanol and eluted with 14 µl of RNase-free water. Small RNA yield has been quantified on the Qubit 2.0 Fluorometer (Life Technologies) and it was around 30-50 ng/mL each sample. 5 µl were converted in cDNA using TaqMan® microRNA Reverse Transcription Kit (Life Technologies) and stem-loop specific RT primers for each selected human miRNA (hsa-miR-1 assay, ID 002222; hsa-miR-133a, assay ID 002246; hsa-miR133b, assay ID 002247; hsa-miR-206, assay ID 000510; hsa-miR-208b, assay ID 002290; hsa-miR-499, assay ID 001045). snRNA U6 was used as endogenous control (assay ID 001973). The mixture was incubated at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Afterwards, quantitative real-time PCR was performed on a QuantStudio3™ Real-Time PCR System (Applied Biosystems) with automatic baseline setting, using TaqMan® Universal Master Mix 2x no UNG (Applied Biosystems), 1 µl 20x Taqman miR Assay (Life Technologies) and 1.33 µl RT

product. Real time reaction has been carried out at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All reactions, including the no-template controls, have been run in triplicate. The relative expression levels of each miRNA in comparison with the normalizer were then calculated using the comparative threshold (Ct) method $2^{-\Delta Ct}$ [39], where ΔCt represents the difference between each miRNA and the normalizer (average Ct for the miRNA minus average Ct for snRNA U6). Expression levels ($2^{-\Delta Ct}$) were log-transformed to better fit a normal distribution.

Statistical analysis

Continuous variables are presented as means and standard deviations (SD), while categorical variables are presented as percentages. Shapiro-Wilk test was used to assess the normality assumption for continuous variable. In case of violation, suitable data transformation methods were adopted for addressing non-normality. Continuous and categorical variables were compared between sarcopenic and non sarcopenic subjects using independent sample t-test or chi-square test as appropriate. A binary logistic regression analysis was used to assess the association between sarcopenia and the variability of the assessed plasma miRNA levels. Multivariate analysis was made after adjustment for variables that were significantly different between sarcopenic and non-sarcopenic subjects, with the exception of those strictly related to sarcopenia diagnosis (HGS, SMI, and Gait speed). Correlation analyses were performed using Spearman's correlation coefficient to determine the magnitude of association between plasma miRNA levels and MNA scores, as well as biochemical markers of nutritional status. Statistical significance was defined as two-tailed p-value <0.05. All statistical analyses were performed using IBM SPSS statistics for Windows, v.25 (IBM Corp).

3. Results

We enrolled a total of 218 subjects with a mean age of 81.6 years of whom 109 (50.0%) were identified as affected by sarcopenia according to the EWGSOP2 criteria. Table 1 shows the baseline characteristics of the study participants, stratified by the presence of sarcopenia. Individuals with sarcopenia were older compared to those without the condition.

Table 1. Anthropometric and biochemical characteristics of participants with and without sarcopenia.

Variables	**No sarcopenia (**N=109**)**	**Sarcopenia (**N=109**)**	**p-value**
Age (yrs)	79.5 (7.3)	83.7 (6.3)	<0.001
Men (%)	39.4	33.0	0.324
HGS (Kg)	22.7 (11.7)	12.4 (5.1)	<0.001

SMI (Kg/m^2)	8.5 (1.8)	6.8 (1.9)	<0.001
Gait speed (m/s)	0.69 (0.33)	0.58 (0.24)	0.094
ADL dependence (>1)	43.7%%	75.9%	<0.001
MNA-SF (<12 pt)	35.4%	59.4%	0.002
MNA-LF (<24 pt)	50.0%	77.4%	0.002
Glucose (mg/dL)	104.3 (33.3)	101 (46.1)	0.555
Total protein (g/dL)	6.6 (0.5)	6.5 (0.7)	0.477
Albumin BCP (g/dL)	54.6 (7.9)	51.8 (6.6)	0.014
Total cholesterol (mg/dL)	169.6 (41.6)	155.4 (39.5)	0.011
Triglycerides (mg/dL)	96.6 (35.1)	85.5 (31.6)	0.025
LDL cholesterol (mg/dL)	51.1 (13.1)	49.2 (14.4)	0.315
HDL cholesterol (mg/dL)	122.3 (79.7)	116.3 (56.2)	0.530
Creatinine (mg/dL)	1.1 (0.3)	1.1 (0.5)	0.961
Uric acid (mg/dL)	4.6 (1.4)	5.6 (7.2)	0.218
Sodium (mM/L)	140.9 (2.6)	140.6 (2.5)	0.459
Potassium (mM/L)	4.4 (0.5)	4.5 (0.6)	0.728
Clorure (mM/L)	104.6 (4.5)	104 (3.7)	0.400
Calcium (mg/dL)	9.2 (0.6)	9.1 (0.6)	0.026
Phosphorus (mg/dL)	3.7 (0.6)	3.7 (1)	0.926
Magnesium (mg/dL)	1.9 (0.3)	1.9 (0.3)	0.243
Iron (μg/dL)	57.7 (29)	53.7 (28.3)	0.402
Ferritin (ng/mL)*	137.4 (177)	204.8 (279.6)	0.036
C-Reactive Protein (mg/L)*	8.9 (12.6)	17.3 (21.9)	0.040

Continuous variables are expressed as mean and standard deviations (SD), while categorical variables are expressed as percentage (%). P value from t-test for contiguous variables and from chi-squared test of association for categorical variables.
*Log-transformed values
Abbreviations: SD, standard deviation; HGS: Hand Grip Strength; ADL: Activities of Daily Living; MNA-SF: Mini Nutritional Assessment Short Form; MNA-LF: MNA Long Form.

Compared with non-sarcopenic subjects, dependency in ADL was more prevalent in sarcopenic subjects (43.7 % *vs* 75.9%, $p < 0.001$). Subjects with sarcopenia who were malnourished according to MNA-SF were 59.4%, whereas only the 35.4% of the non sarcopenic subjects were classified as having a malnutrition status (p=0.002). After the complete assessment of MNA (MNA-LF), the proportion of subjects malnourished and at risk of malnutrition remained significantly higher in the sarcopenic group than in the non-sarcopenic group (77.4% vs. 50.0% p=0.002). Moreover, significant differences in several clinical-biochemical parameters were observed between the two group of subjects (Table 1).

Plasma levels of the myomiRs were investigated in the cohort studied. First, in order to verify the reliability of snRNA U6 as endogenous control to standardize miRNA expression, Ct values of U6

were compared between sarcopenic and non-sarcopenic groups. No significant difference was observed (p=0.528), suggesting that U6 is constitutively expressed in plasma regardless of disease condition, and thus supporting its use as a reliable normalization control. Out of the six miRNAs we analyzed, miR-1, miR-208b and miR-499 were undetected in this sample, while miR-206, miR-133a and miR-133b were found in all subjects.

Univariate analysis ruled out the correlation between age/gender and myomiRs levels ($p > 0.05$).

As reported in Table 2 and in Figure 1, the univariate analysis showed significantly lower plasma levels of miR-133b in subjects with sarcopenia in comparison to non-sarcopenic individuals (model 1, p=0.006).

Table 2. Effect of miRNA-133a, miRNA- 133b and miRNA-206 on sarcopenia according to different logistic regression models.

	Model 1		**Model 2**		**Model 3**	
	OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
miR-133a	1.30 (0.88-1.90)	0.187	1.29 (0.85-1.97)	0.229	1.09 (0.69-1.73)	0.700
miR-133b	0.65 (0.47-0.89)	0.006	0.69 (0.49-0.97)	0.037	0.79 (0.53-1.17)	0.228
miR-206	1.06 (0.80-1.41)	0.675	1.14 (0.84-1.55)	0.413	1.20 (0.86-1.69)	0.288

Model 1: unadjusted ORs
Model 2: ORs adjusted for age and ADL
Model 3: ORs adjusted for age, ADL and MNA-SF (<12)

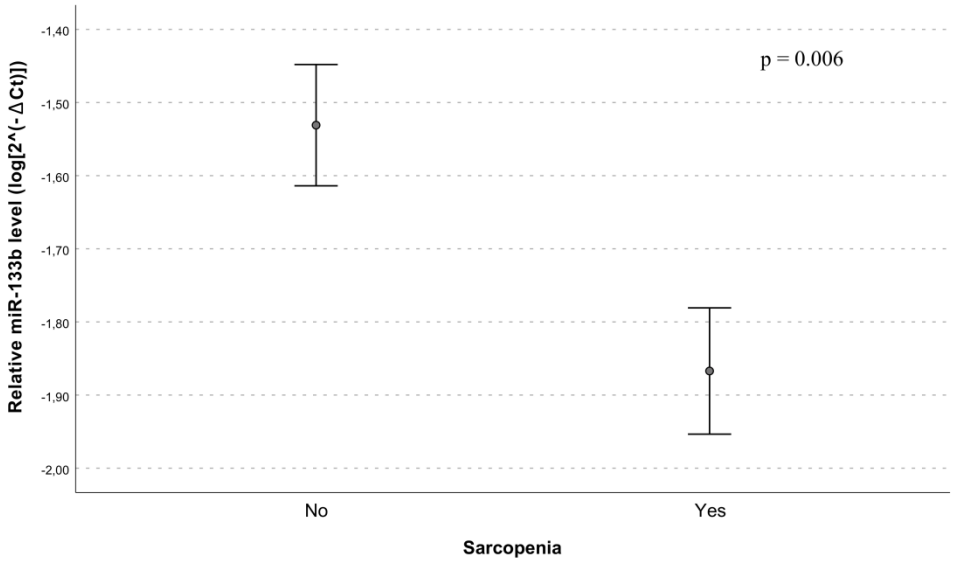

Figure 1. Relative miR-133b expression in plasma from sarcopenic and non-sarcopenic subjects. Data are reported as log $2^{-\Delta Ct}$ normalized to U6 expression together with mean ± standard error of the mean (SEM) and p-value computed by t-test ($p < 0.05$).

After adjusting for age and ADL, the association between miR-133b and sarcopenia remained significant (model 2, p= 0.037) while after additional adjustment for nutritional status, assessed by MNA-SF scores, the effect of miR-133b on sarcopenia disappeared (p=0.238; model 3 in Table 2), suggesting a relationship between this miRNA and nutrition. To get more insights in this relationship we performed correlation analyses of myomiR levels with nutritional status. Data showed that lower levels of both miR-133b and miR-206 were significantly correlated with poor nutritional status assessed either by MNA-SF (miR-133b, rho=0.193, p=0.012; miR-206, rho=0.156, p=0.044) or MNA-LF (miR-133b, rho=0.256, p=0.005; miR-206, rho=0.224, p=0.014) scores. Analysis performed splitting the whole sample by sarcopenia showed that the association between lower levels of both miR-133b and miR-206 and undernutrition was statistically significant in subjects with sarcopenia (Figure 2, A and B). No significant correlation for miR-133a was found.

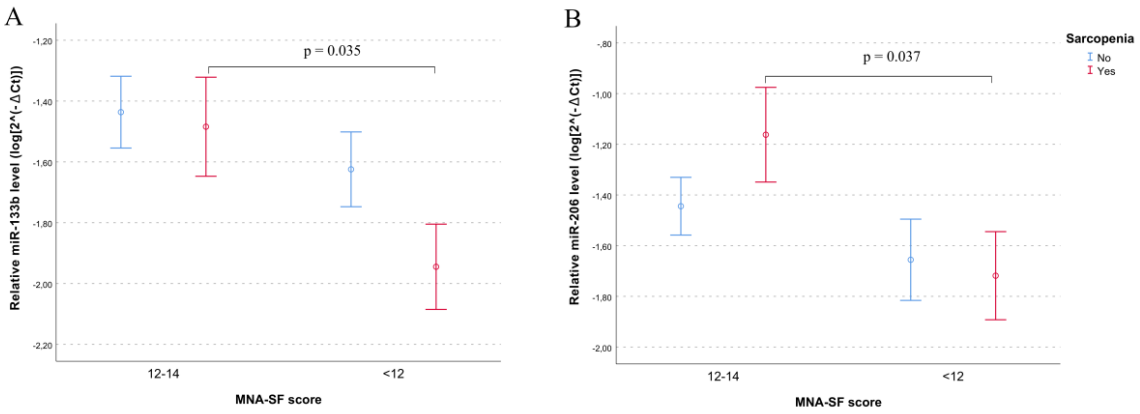

Figure 2. Effect of nutritional status on plasma levels of (**A**) miR-133b and (**B**) miR-206 in sarcopenic and non-sarcopenic subjects. Data are reported as log $2^{-\Delta Ct}$ normalized to U6 expression together with mean ± SEM and p-value computing by t-test ($p < 0.05$).

The association between myomiRs and nutritional status prompted us to assess the association between traditional blood biomarkers of malnutrition and inflammation and the levels of myomiRs, performing additional correlation analyses in the whole sample and in the two sub-groups of subjects with and without sarcopenia. Results, presented in Supplementary Material, Table S1, show a significant positive correlation between the plasma levels of miR-133b and albumin (p<0.001) and serum iron (p=0.003) levels, whereas a negative correlation was observed with ferritin (p= 0.018), in the whole sample of participants. Also, the miR-206 levels were positively and negatively correlated with albumin (p= 0.001) and ferritin (p= 0.014) levels, respectively. As shown by the scatter-plots and linear regressions of Figure 3 and 4, statistically more significant correlations were observed in the group of subjects with sarcopenia for both miR-133b (rho=0.353; P<0.001 for albumin;

rho=0.205; P=0.058 for iron; rho= -0.217; P=0.041 for ferritin; Figure 3) and for miR-206 (rho=0.349; P<0.001 for albumin; rho= -0.187; P=0.077 for ferritin; Figure 4), further highlighting the potential role of nutritional status in mediating the relationship between myomiRs and sarcopenia.

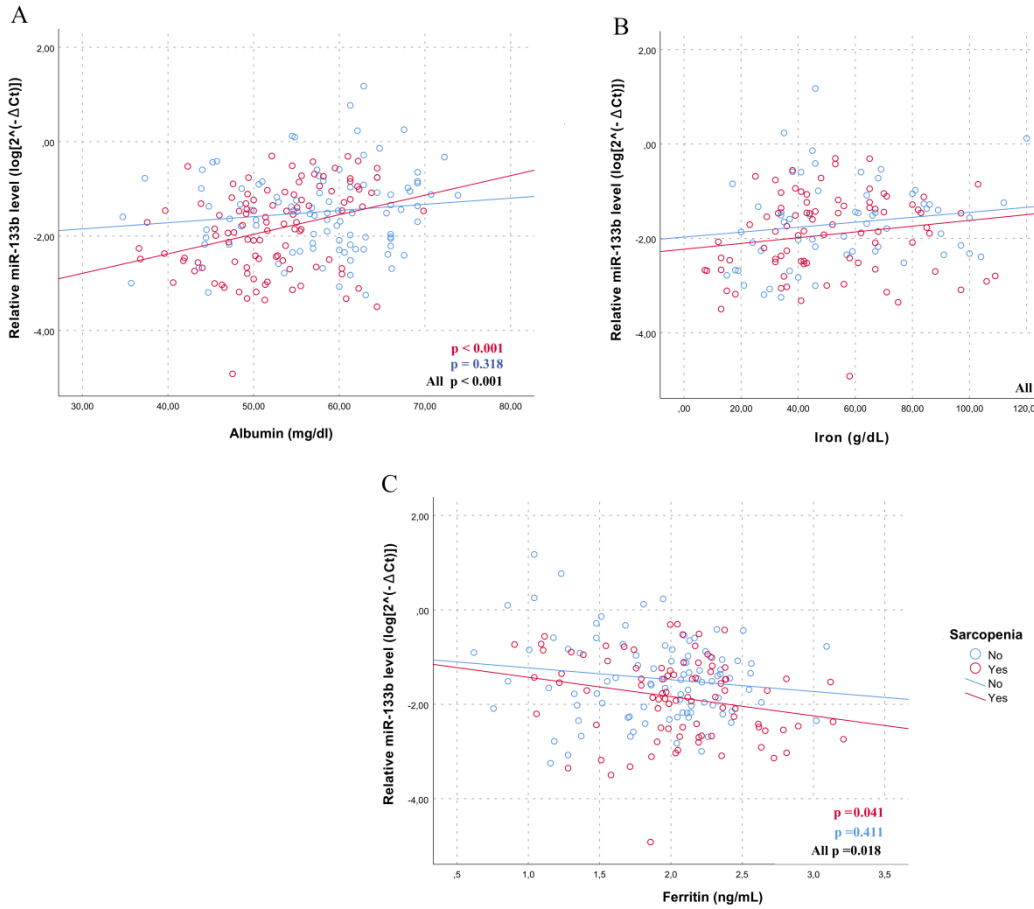

Figure 3. Correlations between plasma miR-133b levels and biochemical variables in sarcopenic and non-sarcopenic subjects. Scatter plots illustrate the relationship between plasma miR-133b levels and (**A**) albumin, (**B**) iron, (**C**) ferritin. Data are reported as log $2^{-\Delta Ct}$ normalized to U6 expression.

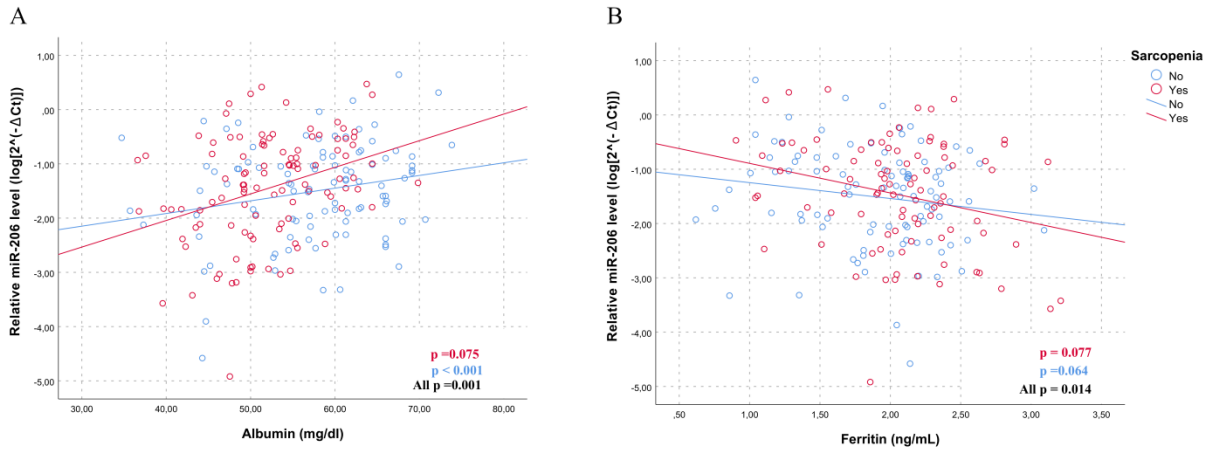

Figure 4. Correlations between plasma miR-206 levels and biochemical variables in sarcopenic and non-sarcopenic subjects. Scatter plots illustrate the relationship between plasma miR-206 levels and (**A**) albumin, (**B**) ferritin. Data are reported as log $2^{-\Delta Ct}$ normalized to U6 expression.

4. Discussion

The present study was set up to assess the relationship among nutritional status, sarcopenia, and levels of myomiRs, muscle-specific miRNAs that affect skeletal muscle processes, from myogenesis to muscle homeostasis.

The analysis of the nutritional status, based on the MNA scores, showed that more than 50% of the participants to our study were malnourished or at risk of malnutrition. This high proportion is not surprising since prevalence among nursing home residents is reported to be higher compared with community-dwelling elders [40]. Anyway, consistent with several reports [41,42,43], we found that the prevalence of subjects malnourished or at risk of malnutrition was significantly higher among those with sarcopenia than those without.

The analysis of the expression profiles of the myomiRs detected in present study (miR-133a, miR133b and miR-206) revealed differential expression of miR-133b between subjects with and without sarcopenia, pointing to a potential role for this miRNA in the disease pathogenesis. MiR-133b regulates fundamental processes of myogenesis including myoblast differentiation, regeneration and satellite cell fate determination [26,44]. Its overexpression has been shown to occur during myogenesis [45], whereas, on the contrary, its downregulation appears to promote satellite cells quiescence, a distinctive feature of sarcopenic muscle which also shows a low regenerative capacity and an impaired differentiation potential [46,47]. Hence, these data, together with ours, support the hypothesis that downregulation of miR-133b may contribute to the decreased myogenic and regenerative capacity of muscle cells, characteristic of sarcopenia. However, when considering the nutritional status of the study population, the association between miR-133b and sarcopenia no

longer remained significant. This suggests that the levels of miR-133b in plasma are correlated with the nutritional status, pointing to a mediating effect of nutrition on the relationship between miR-133b and sarcopenia. Actually, we showed that malnutrition was correlated to lower levels of miR-133b; a similar correlation was found for miR-206. Our study also showed correlations with serum markers, such as albumin and ferritin, more significant in sarcopenic respect to non sarcopenic individuals, thus supporting the evidence that nutritional status may mediate the relationship between sarcopenia and myomiRs.

Skeletal muscle has a significant influence on the metabolic state of the body. The age-related changes that occur within the skeletal muscle alter energy and nutrient metabolism and, in turn, this metabolic dysfunction leads to further deterioration of the skeletal muscle. Diet certainly may well be one of the factors involved in this vicious cycle, as it plays a crucial role in maintaining muscle quality and quantity, both of which have important implications for metabolic capacity and functional performance. Dietary modulation of miRNA expression has been shown influence various diseases, such as cancer, cardiovascular disease, type 2 diabetes and obesity [48]. Accordingly, our findings highlight a nutrient-myomiR pathway that may influence muscle myogenic capacity. In this regard, it is of interest that miR-133a/b and miR-206 appear to be directly or indirectly regulated by the mammalian target of rapamycin (mTOR) [26], the main mediator of cellular nutrient sensing and crucial regulator of skeletal myogenesis and muscle maintenance [49]. Zhang and colleagues [16] proposed a model for nutrients-mTOR-myomiR signalling in skeletal myogenesis, where the kinase-dependent mTOR pathway affects the expression of the above myomiRs through regulation of the myogenic transcription factor MyoD. According to this model, under low nutrient conditions such as amino acids and glucose starvation, mTOR is inactive and unable to induce MyoD synthesis with the consequent down-regulation of miR-133a/b and miR-206. It is also well known, both sarcopenia and malnutrition are related to increased inflammation ad oxidative stress [50,51 ,52 ,53]. Notably, a downregulation of miR-133b and miR-206 was observed in the muscle of patients with inflammatory myopathy [54]. Also, Razak and colleagues reported that treatment with tocotrienol-rich fraction (TRF), known having an antioxidant activity, increases myomiRs expression in myoblasts by reducing the oxidative stress [55]. Here, significant positive and negative correlations were found between miR-133b and miR-206 levels and albumin and ferritin respectively. Notably, decreased albumin and elevated ferritin levels are characteristic features of inflammation besides being markers of nutritional status [56,57 ,58]. Based on these evidence, inflammation, as well as oxidative stress, could represent factors connecting malnutrition, expression of myomiRs and sarcopenia. To this regard, literature data report that inflammation and oxidative stress induce expression of myostatin, a member of the transforming growth factor beta (TGF-β) superfamily that inhibits skeletal muscle

growth muscle mass by downregulating the expression of miR-133b and miR-206 in skeletal muscle [26,50]. In Supplementary Material, Figure S2, we schematically illustrate the possible molecular connections in the tripartite link between poor nutrition, myomiRs and muscle wasting in the elderly. Breafly, poor nutritional status negatively influences mTOR activity, which in turn downregulates myogenic transcription factor MyoD decreasing the expression of miR-133b and miR-206.. Others factors such as oxidative stress and inflammation, interrelated in a vicious circle, can influence the same axes by up-regulating the levels of Myostatin, a repressor of myogenesis, which, in turn, represses myomiRs expression. As a consequence, the downregulation of these miRNAs contributes to muscle wasting by inducing the expression of a number of target genes, some of which have been validated and many more predicted by bioinformatics tools, that should be prioritized and checked in future investigations.

It should be pointed out that, although the sequences of mature miR-133b and miR-133a differ in only one nucleotide at the 3′ end, and that miR-206 and miR-133b constitute a bicistronic cluster on chromosome 6p12.2 (Supplementary Material, Figure S1), in our study miR-133a was not associated with neither nutritional status nor the sarcopenia, as well as miR-206 was not with sarcopenia. This is probably because these myomiRs regulate the myogenic program by activating different downstream targets, and also because their expression is regulated by different upstream signals, likely acting in a context-dependent manner [50,26]. Furthermore, many of the potential targets of myomiRs are not intimately related to myogenic processes, therefore they could be modulated by the state of other interconnected pathways. In addition, myomiRs have emerging roles in the development of a number of non-muscle cells and tissues, beyond their classification as muscle-specific factors. For instance, several reports of myomiR involvement in different types of cancers, as well as in immunological responses and inflammation processes, have emerged in the last years [50].

5. Conclusions

The pathogenesis of sarcopenia is multifactorial, and many of the underlying factors may not act independently in influencing the risk of disease as many of the causal pathways may overlap or interconnect. Our study supports a possible connection among nutrition, expression of miR-133b and miR-206 and age-related skeletal muscle decline; in particular, miR-133b could represent the “trait d’union” between nutritional status and susceptibility to sarcopenia.

However, the conclusions we drawn should be interpreted in the context of limitations of this study. First, ours is a cross-sectional study, so results are limited to revealing associative, rather than causal, relations among nutritional status, miR-133b and miR-206 expression and muscle wasting in

old age. Thus, future investigations should be carried out in order to gain insights about molecular mechanisms underlying the observed associations, exploring both downstream targets and upstream regulators of miR-133b and miR-206 in healthy and sarcopenic subjects, and to evaluate their potential as biomarkers of risk for sarcopenia and malnutrition. Second, along with diet, exercise plays an important role in maintain muscle health because it stimulates protein synthesis. Literature data show that exercise alters the expression pattern of myomiRs, and thus, it would have been also interesting to investigate the effects of exercise on the myomiRs profile in sarcopenic patients. However, in our study, this was not possible because of the lack of information about exercise levels. Therefore, an "ad hoc" study could be designed for the purpose to accurately and comprehensively explore this aspect which may contribute to find preventive strategies and lifestyle changes for reducing the risk of sarcopenia.

References

1. Ahmed, T.; Haboubi, N. Assessment and management of nutrition in older people and its importance to health. *Clin. Interv. Aging* 2010, *5*, 207–216.
2. Dato, S.; Bellizzi, D.; Rose, G.; Passarino, G. The impact of nutrients on the aging rate: A complex interaction of demographic, environmental and genetic factors. Mech Ageing Dev 2016, *154*, 49–61.
3. Agarwal, E.; Miller, M.; Yaxley, A.; Isenring, E. Malnutrition in the elderly: A narrative review. *Maturitas* 2013, *76*, 296–302.
4. Kaiser, M.J.; Bauer, J.M.; Rämsch, C.; Uter, W.; Guigoz, Y.; Cederholm, T.; Thomas, D.R.; Anthony, P.S.; Charlton, K.E.; Maggio, M.; et al. Mini Nutritional Assessment International Group. Frequency of malnutrition in older adults: a multinational perspective using the mini nutritional assessment. *J Am Geriatr Soc* 2010, *58*, 1734–1738.
5. Rasheed, S.; Woods, R. Malnutrition and quality of life in older people: a systematic review and meta-analysis. *Ageing Res Rev* 2013, *12*, 561–566.
6. Covinsky, K.E. Malnutrition and bad outcomes. *J. Gen. Intern. Med* 2002, *17*, 956–957.
7. Harris, D.; Haboubi, N. Malnutrition screening in the elderly population. *J. R. Soc. Med* 2005, *98*, 411–414.
8. Meyer, C.; Dostou, J.M.; Welle, S.L.; Gerich, J.E. Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. *Am J Physiol Endocrinol Metab* 2002, *84*, 475–482.
9. Morley, J.E. Sarcopenia: diagnosis and treatment. *J Nutr Heal. Aging* 2008, *12*, 452–456.
10. Gielen, E.; Verschueren, S.; O'Neill, T.W.; Pye, S.R.; O'Connell, M.D.L.; Lee, D.M.; Ravindrarajah, R.; Claessens, F.; Laurent, M.; Milisen, K.; et al. Musculoskeletal Frailty: A Geriatric Syndrome at the Core of Fracture Occurrence in Older Age. *Calcif. Tissue Int.* 2012, *91*, 161–177.
11. Santilli, V.; Bernetti, A.; Mangone, M.; Paoloni, M. Clinical definition of sarcopenia. *Clin. Cases Miner. Bone Metab.* 2014, *11*, 177–80.
12. Liguori, I.; Russo, G.; Aran, L. Sarcopenia: assessment of disease burden and strategies to improve outcomes. *Clin Interv Aging* 2018, *13*, 913–927.
13. Shafiee, G.; Keshtkar, A.; Soltani, A.; Ahadi, Z.; Larijani, B.; Heshmat, R. Prevalence of sarcopenia in the world: a systematic review and metaanalysis of general population studies. *J Diabetes Metab Disord* 2017, *16*, 21.
14. Bianchi, M.; Renzini, A.; Adamo, S.; Moresi, V. Coordinated Actions of MicroRNAs with other Epigenetic Factors Regulate Skeletal Muscle Development and Adaptation. *Int J Mol Sci* 2017, *18*, 1051–1063.
15. Pillai, R.S. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science (80-.).* 2005, *309*, 1573–1576.
16. Zhang, Y.; Yu, B.; He, J.; Chen, D. From Nutrient to MicroRNA: a Novel Insight into Cell Signaling Involved in Skeletal Muscle Development and Disease. *Int J Biol Sci* 2016, *12*, 1247–1261.
17. Jung, H.J.; Lee, K.P.; Kwon, K.S.; Suh, Y. MicroRNAs in skeletal muscle aging: Current issues and perspectives. *J Gerontol A Biol Sci Med Sci* 2018.

18. Drummond, M.J.; McCarthy, J.J.; Sinha, M. Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis. *Physiol Genomics* 2011, *43*, 595–603.
19. Rivas, D.A.; Lessard, S.J.; Rice, N.P. Diminished skeletal muscle micro-RNA expression with aging is associated with attenuated muscle plasticity and inhibition of IGF-1 signaling. *FASEB J* 2014, *28*, 4133–4147.
20. Margolis, L.M.; Rivas, D.A. Potential role of microRNA in the anabolic capacity of skeletal muscle with aging. *Exerc Sport Sci Rev* 2018, *46*, 86–91.
21. Brown, D.M.; Goljanek-Whysall, K. microRNAs: Modulators of the underlying pathophysiology of sarcopenia? *Ageing Res Rev* 2015, 24(*B)*, 263–273.
22. Güller, I.; Russell, A.P. MicroRNAs in skeletal muscle: their role and regulation in development, disease and function. *J Physiol* 2010, *588*, 4075–4087.
23. McCarthy, J.J. The MyomiR network in skeletal muscle plasticity. *Exerc Sport Sci Rev* 2011, *39*, 150–154.
24. Wang, X.H. MicroRNA in myogenesis and muscle atrophy. *Curr Opin Clin Nutr Metab Care* 2013, *16*, 258–266.
25. Sharma, M.; Juvvuna, P.K.; Kukreti, H.; McFarlane, C. Mega roles of microRNAs in regulation of skeletal muscle health and disease. *Front Physiol* 2014, *5*, 239.
26. Horak, M.; Novak, J.; Bienertova-Vasku, J. Muscle-specific microRNAs in skeletal muscle development. *Dev Biol* 2016, *1*, 1–13.
27. Schiaffino, S.; Mammucari, C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skelet Muscle* 2011, 1(1), 4.
28. Nielsen, S.; Scheele, C; Yfanti, C.; Åkerström, T.; Nielsen, A.R.; Pedersen, B.K.; et al. Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. J Physiol 2010, 588, 4029–4037.
29. Russell, A.P.; Lamon, S.; Boon, H.; Wada, S.; Güller, I.; Brown, E.L.; et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. J Physiol 2013, 591, 4637–4653.
30. Drummond, M.J.; McCarthy, J.J.; Fry, C.S.; Esser, K.A.; Rasmussen, B.B. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. Am J Physiol Endocrinol Metab 2008, 295, E1333-40.
31. Margolis, L.M.; McClung, H.L.; Murphy, N.E.; Carrigan, C.T.; Pasiakos, S.M. Skeletal Muscle myomiR Are Differentially Expressed by Endurance Exercise Mode and Combined Essential Amino Acid and Carbohydrate Supplementation. *Front Physiol* 2017, *23*, 182.
32. Camera, D.M.; Ong, J.N.; Coffey, V.G.; Hawley, J.A. Selective Modulation of MicroRNA Expression with Protein Ingestion Following Concurrent Resistance and Endurance Exercise in Human Skeletal Muscle. Front Physiol. 2016, 7, 87.
33. Haran, P.H.; Rivas, D.A.; Fielding, R.A. Role and potential mechanisms of anabolic resistance in sarcopenia. *J Cachexia Sarcopenia Muscle* 2012, *3*, 157–162.
34. Nielsen, S., Hvid, T.; Kelly, M.; Lindegaard, B.; Dethlefsen, C.; Winding, K.; et al. Muscle specific miRNAs are induced by testosterone and independently upregulated by age. Front Physiol 2014, 4, 394.

35. Janssen, I.; Heymsfield, S.B.; Baumgartner, R.N.; Ross, R. Estimation of skeletal muscle mass by bioelectrical impedance analysis. *J Appl Physiol* 2000, *89*, 465–471.
36. Katz, S.; Downs, T.D.; Cash, H.R.; Grotz, R.C. Progress in Development of the Index of ADL. *Gerontologist* 1970, *10*, 20–30.
37. Vellas, B.; Guigoz, Y.; Garry, P.J.; Nourhashemi, F.; Bennahum, D.; Lauque, S.; Albarede, J.L. The Mini Nutritional Assessment (MNA) and its use in grading the nutritional state of elderly patients. *Nutrition* 1999, *15*, 116–122.
38. Cruz-Jentoft, A.J.; Bahat, G.; Bauer, J.; Boirie, Y.; Bruyère, O.; Cederholm, T.; Cooper, C.; Landi, F.; Rolland, Y.; Sayer, A.A.; et al. Sarcopenia: revised European consensus on definition and diagnosis. *Age Ageing* 2019, *48*, 16–31.
39. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008, 3, 1101–8..
40. Cereda, E.; Pedrolli, C.; Klersy, C. Nutritional status in older persons according to healthcare setting: A systematic review and meta-analysis of prevalence data using MNA((R)) Clin. *Nutr* 2016, *35*, 1282–1290.
41. Lu, Y.; Karagounis, L.G.; Ng, T.P.; Carre, C.; Narang, V.; Wong, G.; Ying Tan, C.T.; Zin Nyunt, M.S.; Gao, Q.; Abel, B.; et al. Systemic and Metabolic Signature of Sarcopenia in Community-Dwelling Older Adults. *J Gerontol A Biol Sci Med Sci* 2019, *8*.
42. Verlaan, S.; Aspray, T.J.; Bauer, J.M.; Cederholm, T.; Hemsworth, J.; Hill, T.R.; McPhee, J.S.; Piasecki, M.; Seal, C.; Sieber, C.C.; et al. Nutritional status, body composition, and quality of life in community-dwelling sarcopenic and non-sarcopenic older adults: A case-control study. *Clin Nutr* 2017, *36*, 267–274.
43. Vandewoude, M.F.; Alish, C.J.; Sauer, A.C.; Hegazi, R.A. Malnutrition-sarcopenia syndrome: is this the future of nutrition screening and assessment for older adults? *J. Aging Res.* 2012, 651570.
44. Cui, S.; Li, L.; Mubarokah, S.N.; Meech, R. Wnt/β-catenin signaling induces the myomiRs miR-133b and miR-206 to suppress Pax7 and induce the myogenic differentiation program. *J Cell Biochem* 2019, *120*, 12740–12751.
45. Feng, Y.; Niu, L.-L.; Wei, W.; Zhang, W.-Y.; Li, X.-Y.; Cao, J.-H.; S.-H. Zhao A feedback circuit between miR-133 and the ERK1/2 pathway involving an exquisite mechanism for regulating myoblast proliferation and differentiation Cell. *Death Dis* 2013, *4*, 934.
46. Alway, S.E. Regulation of Satellite Cell Function in Sarcopenia. *Front Aging Neurosci* 2014, *6*, 246.
47. Sousa-Victor, P.; Muñoz-Cánoves, P. Regenerative decline of stem cells in sarcopenia. *Mol Asp. Med* 2016, *50*, 109–117.
48. Ross, S.A.; Davis, C.D. The emerging role of microRNAs and nutrition in modulating health and disease. *Annu. Rev. Nutr* 2014, *34*, 305–336.
49. Ge, Y.; Chen, J. Mammalian target of rapamycin (mTOR) signaling network in skeletal myogenesis. *J Biol Chem* 2012, *287*, 43928–43935.
50. Mitchelson, K.R.; Qin, W.Y. Roles of the canonical myomiRs miR-1, -133 and -206 in cell development and disease. *World J Biol Chem* 2015, *6*, 162–208.
51. Dalle, S.; Rossmeislova, L.; Koppo, K. The Role of Inflammation in Age-Related Sarcopenia. *Front Physiol* 2017, *8*, 1045.

52. Aly, G.S.; Shaalan, A.H.; Mattar, M.K. Oxidative stress status in nutritionally stunted children. *Gaz Egypt Paediatr Assoc* 2014, 28–33.
53. Mueller, C. Inflammation and Malnutrition. *Top. Clin. Nutr.* 2011, *26*, 3–9.
54. Georgantas, R.W.; Streicher, K.; Greenberg, S.A.; Greenlees, L.M.; Zhu, W.; Brohawn, P.Z.; Higgs, B.W.; Czapiga, M.; Morehouse, C.A.; Amato, A.; et al. Inhibition of myogenic microRNAs 1, 133, and 206 by inflammatory cytokines links inflammation and muscle degeneration in adult inflammatory myopathies. *Arthritis Rheumatol* 2014, *66*, 1022–1033.
55. Razak, A.M.; Khor, S.C.; Jaafar, F.; Karim, N.A.; Makpol, S. Targeting myomiRs by tocotrienol-rich fraction to promote myoblast differentiation. *Genes Nutr* 2018, *13*.
56. Keller, U. Nutritional Laboratory Markers in Malnutrition. *J Clin Med* 2019, *8*, 775.
57. Soeters, P.B.; Wolfe, R.R.; Shenkin, A. Hypoalbuminemia: Pathogenesis and Clinical Significance. *J Parenter Enter. Nutr* 2018, *43*.
58. Moen, I.W.; Bergholdt, H.K.M.; Mandrup-Poulsen, T. Increased Plasma Ferritin Concentration and Low-Grade Inflammation. A Mendelian Randomization Study. *Clin Chem* 2018, *64*.

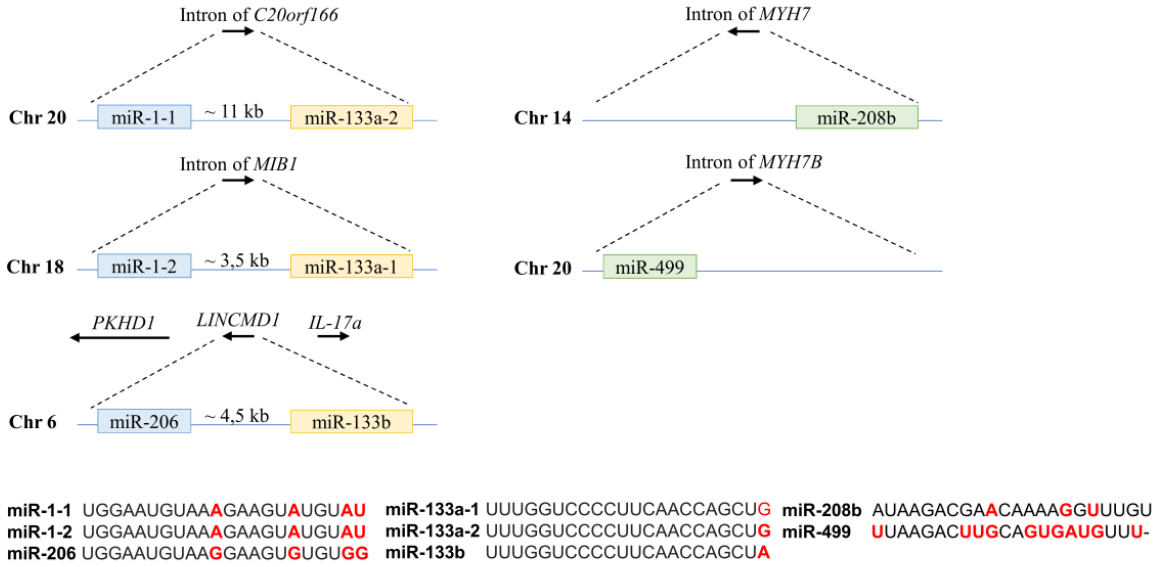

Figure S1. Genomic structure of human muscle-specific microRNAs (myomiRs), host genes and myomiRs sequence homologies.

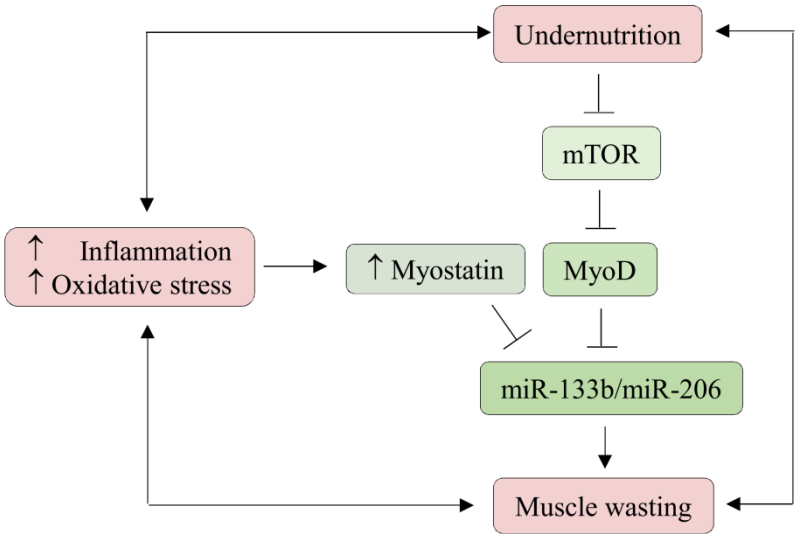

Figure S2. Schematic illustration of the hypothetical molecular connections in the tripartite link between poor nutrition, myomiRs and muscle wasting in the elderly. Poor nutritional status negatively influences mTOR activity, which in turn downregulates myogenic transcription factor MyoD decreasing the expression of miR-133b and miR-206, potentially leading to muscle wasting. Others factors such as oxidative stress and inflammation, interrelated in a vicious circle, can influence the same axes by up-regulating the levels of Myostatin, a repressor of myogenesis, which, in turn, represses myomiRs expression, so contributing to muscle wasting too.

Table S1. Correlations between plasma levels of miR-133a, -133b and -206 and biochemical variables in sarcopenic and non-sarcopenic individuals.

miR-133a	**Non sarcopenic**		**Sarcopenic**		**Total**	
	rho	**p-value**	**rho**	**p-value**	**rho**	**p-value**
Glucose (mg/dL)	-0.016	0.869	0.117	0.233	0.034	0.622
Total protein (g/dL)	-0.082	0.505	0.007	0.947	-0.022	0.779
Albumin (%)	0.011	0.916	0.196	0.046	0.037	0.597
Total cholesterol(mg/dL)	0.028	0.776	-0.148	0.128	-0.080	0.248
Tryglicerid (mg/dL)	-0.046	0.650	0.067	0.496	-0.011	0.876
LDL cholesterol (mg/dL)	0.058	0.577	-0.157	0.137	-0.052	0.483
HDL cholesterol (mg/dL)	-0.141	0.163	-0.134	0.182	-0.132	0.061
Creatinine (mg/dL)	-0.120	0.218	-0.022	0.821	-0.076	0.268
Uric acid (mg/dL)	-0.103	0.372	-0.117	0.260	-0.095	0.216
Sodium (mM/L)	-0.029	0.767	0.001	0.994	-0.015	0.825
Potassium (mM/L)	0.150	0.127	0.031	0.752	0.101	0.144
Clorure (mM/L)	-0.029	0.823	0.129	0.234	0.069	0.404
Calcium (mg/dL)	0.052	0.614	0.065	0.508	0.036	0.609
Phosphorus (mg/dL)	-0.016	0.876	0.100	0.350	0.007	0.923
Magnesium (mg/dL)	-0.193	0.109	-0.041	0.708	-0.121	0.132
Iron (μg/dL)	0.031	0.811	0.012	0.913	0.024	0.770
Ferritin (ng/mL)*	-0.087	0.404	-0.173	0.102	-0.129	0.081
C-Reactive Protein (mg/L)*	-0.013	0.919	-0.042	0.710	-0.033	0.693
miR-133b						
	Non sarcopenic		**Sarcopenic**		**Total**	
	rho	**p-value**	**rho**	**p-value**	**rho**	**p-value**
Glucose (mg/dL)	-0.168	0.091	-0.061	0.535	-0.093	0.182
Total protein (g/dL)	-0.093	0.453	-0.026	0.798	-0.029	0.717
Albumin (g/dL)	0.102	0.318	0.353	**<0.001**	0.265	**<0.001**
Total cholesterol (mg/dL)	0.079	0.423	0.148	0.130	0.148	0.132
Tryglicerid (mg/dL)	-0.105	0.293	0.034	0.734	-0.025	0.723
LDL cholesterol (mg/dL)	0.064	0.537	0.122	0.254	0.131	0.076
HDL cholesterol (mg/dL)	0.040	0.693	0.045	0.656	0.066	0.357
Creatinine (mg/dL)	-0.034	0.726	-0.073	0.454	-0.057	0.403
Uric acid (mg/dL)	-0.068	0.558	-0.169	0.103	-0.134	0.082

Sodium (mM/L)	0.158	0.109	-0.014	0.891	0.076	0.275
Potassium (mM/L)	0.046	0.640	-0.130	0.187	-0.042	0.542
Clorure (mM/L)	0.133	0.377	0.102	0.350	0.114	0.168
Calcium (mg/dL)	0.077	0.450	0.096	0.325	0.111	0.114
Phosphorus (mg/dL)	0.048	0.640	0.185	0.082	0.124	0.092
Magnesium (mg/dL)	-0.031	0.796	-0.009	0.934	0.006	0.940
Iron (μg/dL)	0.232	0.072	0.205	0.058	0.239	**0.003**
Ferritin (ng/mL)*	-0.086	0.411	-0.217	**0.041**	-0.175	**0.018**
C-Reactive Protein (mg/L)*	-0.075	0.546	-0.101	0.375	-0.110	0.185
miR-206						
	Non sarcopenic		**Sarcopenic**		**Total**	
	rho	**p-value**	**rho**	**p-value**	**rho**	**p-value**
Glucose (mg/dL)	0.095	0.337	-0.024	0.805	0.020	0.779
Total protein (g/dL)	-0.188	0.124	0.147	0.151	0.023	0.766
Albumin (g/dL)	0.181	0.075	0.349	**<0.001**	0.223	**0.001**
Total cholesterol (mg/dL)	-0.106	0.280	-0.013	0.893	-0.063	0.359
Tryglicerid (mg/dL)	-0.211	0.034	-0.051	0.605	-0.127	0.069
LDL cholesterol (mg/dL)	-0.072	0.490	-0.054	0.613	-0.073	0.322
HDL cholesterol (mg/dL)	-0.060	0.556	0.077	0.448	0.005	0.945
Creatinine (mg/dL)	0.064	0.511	0.017	0.863	0.042	0.540
Uric acid (mg/dL)	0.073	0.530	-0.075	0.468	-0.009	0.912
Sodium (mM/L)	-0.054	0.589	0.049	0.619	-0.002	0.982
Potassium (mM/L)	0.043	0.662	0.175	0.073	0.117	0.090
Clorure (mM/L)	-0.026	0.840	0.104	0.336	0.051	0.534
Calcium (mg/dL)	-0.067	0.509	0.237	0.014	0.086	0.223
Phosphorus (mg/dL)	0.111	0.279	0.130	0.223	0.108	0.140
Magnesium (mg/dL)	-0.092	0.451	0.079	0.472	-0.006	0.943
Iron (μg/dL)	-0.228	0.078	0.061	0.573	-0.053	0.524
Ferritin (ng/mL)*	-0.191	0.064	-0.187	0.077	-0.181	**0.014**
C-Reactive Protein (mg/L)*	0.110	0.374	-0.123	0.276	-0.038	0.647

*Log-transformed values

CHAPTER 7

General Discussion

Aging and longevity are extremely heterogeneous phenotypes resulting from a complex interaction among genetics, epigenetics, and the environmental factors. This heterogeneity and complexity have triggered a continuous growth of the research on healthy and unhealthy aging in the last decades, trying to enlarge the time an individual live in good health, possibly delaying age-related diseases, and so reaching longevity. In such a scenario, one of the main goals is the definition of novel genetic and epigenetic markers that shape the individual variability in aging and lifespan. This PhD thesis aims to contribute to this goal.

In **Chapter 2**, the analysis of genetic variants of *XME* (xenobiotic-metabolizing enzymes) genes highlighted that the genetic variability of these genes may affect the chance of reaching old ages, most likely by affecting the dynamic and complex gene–environment interactions. Thus, these findings provide further support to the view that the body's ability to respond to internal and external exposome is crucial for survival. Interestingly, the associated SNPs showed age-specific effects on longevity, showing different trends of XME gene frequencies with age (either linear or non-linear trajectories), that once again highlight the complexity in gene-longevity associations

As discussed in the introduction to this thesis, age-specific gene effects have already been reported in literature for several genetic variants. Recently, this issue has been treated by Giuliani et al (2018) who analyzed the allele frequencies trajectories in an Italian cohort divided in different age subgroups and showed that the change of allele frequency with age may follow linear trends (monotonic) or non-monotonic patterns (usually U-shaped patterns or constant trends until a certain age and then linear ones), in which allele frequency decreases at a given age, but then increases, thus reflecting the establishment of trade-offs in the effect of variants at young and old ages (Ukraintseva et al, 2016). SNPs showing higher allele frequencies in 50-year-old group than younger and centenarians are called "from good to bad" SNPs, because they show beneficial effects in middle life but not for longevity. Other SNPs, the so called “from bad to good”, showed lower allele frequency in 50-year-old group and higher in centenarians and are supposed to have a beneficial effect for longevity (Giuliani et al, 2018).

The variants of *XME* genes that we associated with longevity surely can fall into these categories. For instance, rs3745274-T in *CYP2B6* falls into the category of SNPs “from good to bad” thus acting as a killing SNP, whereas rs776746-A in *CYP3A5*, rs4680-AA in *COMT*, and rs2273697-A in *ABCC2* can be included into the category “from bad to good”, thus acting as pro-longevity SNPs. In particular, rs2273697-A showed a U-like frequency curve, a behavior typical of variants which act according to a buffering mechanism as suggested by Bergman and colleagues (2007), stating that a deleterious effect of a genetic variant can be neutralized by the protective effect of pro-longevity genes.

These frequency trends are likely correlated to the age-related changes in metabolism, body composition and hormonal profile, which can alter the internal milieu in which genes work. In such a changing environment, genes will not necessarily have a uniform effect on the same phenotype at all ages, and a risk factor for some disease in a middle-aged body may not always be the risk factor in an older body (Ukraintseva et al, 2016).

The found "good" and "bad" effects on longevity agree with literature studies which highlighted the influence of these *XME* variants on susceptibility to the risk of multiple cancers (Justenhoven et al, 2014) and on the health of the cognitive status (Wang et al, 2016), thus supporting their effects on survival. To this regard it is intriguing that the highest impact of the above variants was observed in the age range 65-89 years, which is the age range mostly characterized by the development and progression of age-related diseases.

Also, an important finding was that the genetic variability of these genes accounts for 7.7% of the chance to survive beyond the age of 89 years. This result is significant if we consider that about 25% of the variation in human longevity is due to genetic factors (Passarino et al, 2016).

On the whole, our study gives additional hints to the knowledge of the genetic components that modulate longevity, highlighting that *XME* genes can be useful genetic markers of aging and longevity.

In **Chapter 3**, we provided evidence about the influence of the variability at the *IPMK* (Inositol polyphosphate multikinase) locus on survival. It should be noted that this study is the first to analyze the association of *IPMK* variability with aging and longevity. What prompted us to the choice of this candidate gene has been the multifunctional features of the encoded protein. In fact, IPMK itself can be considered as a moonlighting protein because, as enzyme, it catalyzes the synthesis of inositol pyrophosphates, secondary messengers acting in several aspects of cell physiology, whereas, by a direct protein-protein interaction and independently from its enzymatic activity, it acts as signaling hub in regulating nutrient and energetic pathways (Resnick et al, 2008). Our work hypothesis is that analyzing genes with a role in multiple networks and biological mechanisms may help to have a broader ranging analytical approach which is important when treating with extreme complex phenotypes such as aging and longevity.

In our study, six polymorphisms of *IPMK* resulted to significantly affect the female chance of survival to old age, thus showing a gender-specific effect.

Several genes involved in determining life span have been found to have different influences on the probability of achieving longevity in men and women (Zeng et al, 2018). Gender-specific effects were observed in linkage analysis, with a male-specific linkage peak at 8p and female-specific ones at 15q and the 19q *APOE* locus (Beekman et al, 2013). Gender-specific longevity alleles have also

been confirmed by genome-wide association studies (GWAS). Indeed, a study of 2178 cases and 2299 controls identified 35 male-specific and 25 female-specific longevity loci (Zeng et al, 2018). Authors also reported eleven male-specific pathways involving inflammation and immunity genes and 34 female-specific pathways (tryptophan metabolism and PGC-1α induced) that were significantly associated with longevity ($P < .005$) (Zeng et al, 2018), thus suggesting that different pathways contribute to longevity in men and women. It is interesting to note that many members of these pathways are known to perform diverse unrelated functions, behaving as multifunctional proteins. Moreover, it is intriguing that many of the processes involving IPMK influence aging in a gender-specific manner, and that association of *IPMK* variants with Alzheimer's disease were found in women, as reported in **Chapter 4**.

Differences in genetic structure may in part account for the gender difference in life expectancy. As reported in literature data, females live longer than males and, examining data from the Gerontology Research Group, women comprise 90% of supercentenarians that is individuals living to 110 years or longer (Austad et al, 2016). However, despite women's survival advantage, they suffer greater morbidity particularly late in life (Austad et al, 2016). This phenomenon is known as the mortality-morbidity paradox. Overall, sex difference in longevity has been attributed to a combination of genetic factors, environmental and socio-cultural factors. The fact that women have 2 X chromosomes may provide advantageous redundancy because women have a second X to compensate for a mutation, whereas men do not. Female longevity advantage may result also from hormonal influences on inflammatory and immunological responses or greater resistance to oxidative damage (Villa et al, 2015). The rise and decline of estrogen levels in female body with age could be an example of the change in internal environment, which can lead to the shift from bad to good of risk variants, as expressed in **Chapter 2**. In particular, the higher estrogen levels at the reproductive period may increase risks of certain pathologies such as cancer, but at menopause this risk declines promoting female survival and contributing to gender differences.

Based on this evidence, from our study *IPMK* emerged as a novel gender-specific genetic determinant of human longevity and its variants may be added to other identified gender-specific longevity alleles traceable in literature. Moreover, this work may represent the basis to further studies to clarify the different mechanisms underlying male and female longevity and encourages the detection of multifunctional proteins like IPMK which can represent crucial factors in the complex connections among aging, health, and longevity.

From a genetic point of view, this complex connection comes out clearly from the study reported in **Chapter 4**, where we investigated the variability of *IPMK* and *IP6K3*, another gene involved in the inositol pyrophosphates synthesis and in several crucial physiological processes, in relation to risk of

Late Onset Alzheimer Disease (LOAD) and longevity. Indeed, we found that an allele within the *IP6K3* locus (rs10947435-A) previously associated with increased risk for LOAD (Crocco et al, 2016) also increased the chance to become long-lived, while a subset of alleles at *IPMK* locus (rs2790156-A, rs2790234-G, rs2590320-A, rs2251039-T), that we have found to decrease the chance to become long-lived in the study from **Chapter 3**, decreased the risk for LOAD. The phenotype-specific interactions found among different markers of *IPMK* and *IP6K3* strongly suggest that SNP-SNP interactions play a role in these puzzling associations, supporting the notion that epistatic interactions significantly contribute to the non-additive heritability of complex trais as neurodegeneration and longevity (Mackay, 2014).

Also, the study fosters the hypothesis that genes promoting longevity and/or affecting disease risks may be found in hubs interconnecting several signaling pathways. This could be particularly true for genes, such as *IP6K3* and *IPMK*, which participate in very different biological processes that require the coordinate action of distinct signalling network.

Therefore, from studies in **Chapters 3 and 4**, the multifunctionality of the proteins is proposed as the high road to disentangle the complexity and heterogeneity of healthy/unhealthy aging and longevity, and their inter-relationships.

As a proof of the concept that genetic variants may or may not have puzzling behaviours in influencing lifespan, in **Chapter 5** we provided evidence of association between the Longevity associated variant (LAV) of *BPIFB4* and reduced frailty in humans. LAV-*BPIFB4* was already screened in a multi-step genetic analysis showing the homozygous enrichment of LAV haplotype in long-lived individuals (Villa et al, 2015a). Moreover, long-lived individuals expressed higher BPIFB4 transcript as compared to younger controls, corroborating a possible protective role of the protein. *In vitro* and *in vivo* experiments pointed to a role of the protein in survival processes, such as stress response, proteostasis, genomic integrity, eNOS activation, endothelial function, revascularization (Villa et al, 2015a). All these aspects are lost during aging whereas can be kept active in long-lived individuals by higher expression of the BPIFB4 protein. To the best of our knowledge, this is the first study to present both clinical and experimental evidence about the influence of *BPIFB4* haplotypes on frailty in human. Studies in model organisms demonstrated the LAV benefits on endothelial function and eNOS activity: for instance, in hypertensive rats and old mice, gene transfer of LAV-*BPIFB4* restored endothelial nitric oxide synthase signaling, rescued endothelial dysfunction, and reduced blood pressure levels (Villa et al, 2015b). Endothelial dysfunction has been linked to frailty since poor circulation could compromise the ability of an old organism to cope with stress, thus increasing vulnerability. Moreover, the increase in oxidative stress occurring in frail individuals, coupled with the reduction in nitric oxide production, may generate a

reduction in nitric oxide bioavailability, thereby inducing endothelial dysfunction (Mansur et al, 2015). Based on this evidence and our findings, it is possible to hypothesize that LAV haplotype can contribute to halt frailty by protecting vasculature.

Among predisposing factors for frailty, both atherosclerosis and inflammatory processes appear as central hubs. Indeed, Veronese and colleagues found that frail people had elevated inflammatory markers, particularly c-reactive protein, which corresponded also to higher markers of thrombosis (Veronese et al, 2017). Moreover, frail participants also showed a higher presence of carotid plaques (Veronese et al, 2017). In this regard, gene therapies showed that LAV-*BPIFB4* counteracted the vascular atherosclerosis and exerted anti-inflammatory effects (Puca et al, 2020).

Therefore, our results of lower frailty index in mice injected with LAV agree with literature data and it is tempting to speculate that LAV may contrast the low-grade chronic inflammation thus showing the protective effect on frailty.

Overall, our study suggests that variants involved in lifespan can be also screened for age-related diseases (as seen also in **Chapter 2 and 4**), highlighting *BPIFB4* as a potential useful genetic marker of frailty.

The importance of health span instead of life span has gained substantial recognition over the past decade. Health span is defined as the period of life spent in relatively good health. This definition carries with it the necessity to look for biomarkers of healthy/unhealthy aging. Within this frame, the second objective of this PhD thesis was the search of epigenetic markers of quality of aging. In particular, as reported in **Chapter 5,** to achieve this goal, we investigated the relationship among muscle-specific microRNAs (myomiRs), sarcopenia, considered the biological substrate for the development of physical frailty, and malnutrition, a condition strongly related to the decreased quality of aging.

Many studies investigated expression changes of miRNAs in muscles and/or blood (serum or plasma) of patients with sarcopenia and reported that modulation of miRNAs has a critical impact on phenotypes of sarcopenia such as lower physical functions, and on the expression levels of many signalling molecules that mediate disease progression (Yanai et al, 2020 and references there in). However, no study investigated the expression changes of plasma/serum myomiRs in sarcopenia in humans.

From our study it emerged that the expression levels of miR-133b in subjects with sarcopenia were lower then those with non-sarcopenia, indicating a potential role of this miRNA in the disease pathogenesis. As supported by literature evidence which show that miR-133b regulates myogenesis and satellite cell fate determination (Horak et al, 2016), the downregulation of this miRNA could

contribute to satellite cells quiescence and decreased myogenesis, which are distinctive feature of sarcopenic muscle (Alway et al, 2014).

Interestingly, the association between this myomiR and sarcopenia was not independent from nutritional status, indicating that the relationship between miR-133b and sarcopenia is affected by nutrition, a finding supported by the correlations we found with serum markers of nutritional status. Similar correlations were also found for miR-206. Thus, the results from this study highlighted a nutrient-myomiR pathway which may influence muscle myogenic capacity, and then affect muscle functionality. In particular, miR-133b emerged as the "trait d'union" between nutritional status and susceptibility to sarcopenia, and as a potential non-invasive biomarker of muscle damage in malnourished subjects.

The relationship existing between nutrition and human health is quite complex. In elderly, the gradual decline in energy intake, in particular the imbalance between energy from carbohydrates (increased), fat (decreased) and protein (decreased), causes measurable adverse effects on tissue/body form (body shape, size and composition) and function, and clinical outcome (De Groot et al, 2010). Protein intake imbalance mostly has direct effects on skeletal muscle because of its role as a reservoir of AAs (that can support energy production and protein synthesis). The above relationship is significant, considering that nutritional components have a regulatory role in physiological processes crucial for cell survival such as inflammation or immune function (Santoro et al, 2014).

In many malnourished patients there is an associated inflammation, with a complex interplay between the two, where inflammation promotes malnutrition and adverse outcomes by provoking anorexia, by altering metabolism with elevation of resting energy expenditure and by increasing muscle catabolism (Jensen, 2015). On the basis of our evidence of a correlation of myomiR levels with serum levels of albumin and ferritin, which are both markers of nutritional status and characteristic features of inflammation (Keller, 2019; Moen et al, 2018), we can argue that inflammation could in part explain the connections we observed among malnutrition, expression of myomiRs, and sarcopenia.

Thus, by combining our findings and literature data, we proposed a model to try to explain the molecular connections in the tripartite link between poor nutrition, myomiRs, and muscle wasting in the elderly. In such a model, nutritional status, nutrient-sensing pathways, myogenic factors, myiomiRs and inflammation act in a vicious circle leading to muscle wasting.

In future, a system biology data integration approach may provide insight about the validity of this model to explain the relationship among myomiRs, sarcopenia and malnutrition.

Conclusions

Overall, the obtained results of this PhD thesis contribute to untangle the complex connections among aging, health, and longevity, confirming that genetic and epigenetic determinants can influence healthy/unhealthy aging in sex- and age-specific way and interacting with the environment. In future, a better definition of the aging phenotype, combining study designs, as well as the use of novel methods and technologies, multimarker prediction and an integrated systemic approach, may help to identify novel loci, upstream regulators and downstream targets of microRNAs and to recognize the most relevant profiles and pathways involved in healthy/unhealthy aging and longevity.

References

1. Alway SE, Myers MJ, Mohamed JS. Regulation of satellite cell function in sarcopenia. Front Aging Neurosci. 2014;6:246.
2. Austad SN, Fischer KE. Sex Differences in Lifespan. Cell Metab. 2016;23(6):1022-1033.
3. Beekman M, Blanché H, Perola M, et al. Genome-wide linkage analysis for human longevity: Genetics of Healthy Aging Study. Aging Cell. 2013;12(2):184-193.
4. Bergman A, Atzmon G, Ye K, et al. Buffering mechanisms in aging: a systems approach toward uncovering the genetic component of aging. PLoS Comput Biol. 2007;3(8):e170.
5. Crocco P, Saiardi A, Wilson MS, Maletta, et al. Contribution of polymorphic variation of inositol hexakisphosphate kinase 3 (IP6K3) gene promoter to the susceptibility to late onset Alzheimer's disease. Biochim Biophys Acta. 2016;1862(9):1766–1773.
6. De Groot CP, van Staveren WA. Nutritional concerns, health and survival in old age. Biogerontology. 2010;11(5):597-602.
7. Giuliani C, Sazzini M, Pirazzini C, et al. Impact of demography and population dynamics on the genetic architecture of human longevity. Aging (Albany NY). 2018;10(8):1947-1963.
8. Horak M, Novak J, Bienertova-Vasku J. Muscle-specific microRNAs in skeletal muscle development. Dev Biol. 2016;410(1):1-13.
9. Jensen GL. Malnutrition and inflammation-"burning down the house": inflammation as an adaptive physiologic response versus self-destruction? JPEN J Parenter Enteral Nutr. 2015;39(1):56-62.
10. Justenhoven C, Pentimalli D, Rabstein S, et. al. CYP2B6*6 is associated with increased breast cancer risk. Int J Cancer. 2014; 134(2):426-30.
11. Keller U. Nutritional Laboratory Markers in Malnutrition. J Clin Med. 2019;8(6):775.
12. Mackay T. F. Epistasis and quantitative traits: using model organisms to study gene-gene interactions. Nature reviews. Genetics. 2014;15(1):22–33.
13. Mansur HN, Lovisi JC, Colugnati FA, et al. Association of frailty with endothelial dysfunction and its possible impact on negative outcomes in Brazilian predialysis patients with chronic kidney disease. BMC Nephrol. 2015;16:157.
14. Moen IW, Bergholdt HKM, Mandrup-Poulsen T, et al. Increased Plasma Ferritin Concentration and Low-Grade Inflammation-A Mendelian Randomization Study. Clin Chem. 2018;64(2):374-385.
15. Passarino G, De Rango F, Montesanto A. Human longevity: Genetics or Lifestyle? It takes two to tango. Immun Ageing. 2016; 13:12.
16. Puca AA, Carrizzo A, Spinelli C, et al. Single systemic transfer of a human gene associated with exceptional longevity halts the progression of atherosclerosis and inflammation in ApoE knockout mice through a CXCR4-mediated mechanism. Eur Heart J. 2020;41(26):2487-2497.
17. Resnick AC, Saiardi A. Inositol polyphosphate multikinase: metabolic architect of nuclear inositides. Front Biosci. 2008;13:856-66.
18. Santoro A, Pini E, Scurti M, et al. Combating inflammaging through a Mediterranean whole diet approach: the NU-AGE project’s conceptual framework and design. Mech Ageing Dev. 2014;136–137:3–13.Ukraintseva S, Yashin A, Arbeev K, et al. Puzzling role of genetic risk factors in human longevity: "risk alleles" as pro-longevity variants. Biogerontology. 2016;17(1):109-127.
19. Veronese N, Sigeirsdottir K, Eiriksdottir G, et al. Frailty and Risk of Cardiovascular Diseases in Older Persons: The Age, Gene/Environment Susceptibility-Reykjavik Study. Rejuvenation Res. 2017;20(6):517-524.

20. Villa A, Rizzi N, Vegeto E, et al. Estrogen accelerates the resolution of inflammation in macrophagic cells. Sci Rep. 2015;5:15224.
21. Villa F, Carrizzo A, Spinelli CC, et al. Genetic Analysis Reveals a Longevity-Associated Protein Modulating Endothelial Function and Angiogenesis. Circ Res. 2015b;117(4):333-345.
22. Villa F, Malovini A, Carrizzo A, et al. Serum BPIFB4 levels classify health status in long-living individuals. Immun Ageing. 2015a;12:27.
23. Wang M, Ma Y, Yuan W, et al. Meta-Analysis of the COMT Val158Met Polymorphism in Major Depressive Disorder: Effect of Ethnicity. J Neuroimmune Pharmacol. 2016; 11(3):434-45.
24. Yanai K, Kaneko S, Ishii H, et al. MicroRNAs in Sarcopenia: A Systematic Review. Front. Med. 2020;7:180.
25. Zeng Y, Nie C, Min J, et al. Sex Differences in Genetic Associations With Longevity. JAMA Netw Open. 2018;1(4):e181670.

List of publications

Full papers:

Crocco P, Montesanto A, Dato S, Geracitano S, **Iannone F**, Passarino G, Rose G. Inter-Individual Variability in Xenobiotic-Metabolizing Enzymes: Implications for Human Aging and Longevity. Genes (Basel). 2019;10(5):403.

De Rango F, Crocco P, **Iannone F**, Saiardi A, Passarino G, Dato S, Rose G. Inositol Polyphosphate Multikinase (*IPMK*), a Gene Coding for a Potential Moonlighting Protein, Contributes to Human Female Longevity. Genes (Basel). 2019;10(2):125.

Malavolta M, Dato S, Villa F, De Rango F, **Iannone F**, Ferrario A, Maciag A, Ciaglia E, D'amato A, Carrizzo A, Basso A, Orlando F, Provinciali M, Madeddu P, Passarino G, Vecchione C, Rose G, Puca A. LAV-*BPIFB4* associates with reduced frailty in humans and its transfer prevents frailty progression in old mice. Aging (Albany NY). 2019;11(16):6555-6568.

Dato S, Hoxha E, Crocco P, **Iannone F**, Passarino G, Rose G. Amino acids and amino acid sensing: implication for aging and diseases. Biogerontology. 2019;20(1):17-31.

Iannone F, Montesanto A, Cione E, Crocco P, Caroleo MC, Dato S, Rose G, Passarino G. Expression Patterns of Muscle-Specific miR-133b and miR-206 Correlate with Nutritional Status and Sarcopenia. Nutrients. 2020;12(2):297.

Dato S, Crocco P, De Rango F, **Iannone F**, Maletta F, Bruni AC, Saiardi A, Rose G, Passarino G. *IP6K3* and *IPMK* variations in LOAD and longevity: evidence for a multifaceted signaling network at the crossroad between neurodegeneration and survival. Mechanisms of Ageing and Development. 2021; 111439.

Book chapters:

Bellizzi D, Guarasci F, **Iannone F**, Passarino G, Rose G. Centenarians – An Example of Positive Biology. Chapter 7: Epigenetics and Ageing. Editor C. Caruso. Springer 2019.

Crocco P, Dato S, **Iannone F**, Passarino G Rose G. Different components of frailty in the aging subjects: The role of sarcopenia, *submitted*

Congress abstracts:

Iannone F, Montesanto A, Crocco P, Geracitano S, Maletta R, Bruni AC,Passarino G, Rose G. (2018). “Disfunzione mitocondriale e degenerazione neuronale: un polimorfismo del gene UCP4 modula il rischio di Alzheimer e Malattia Frontotemporale”. Congresso per il Ventennale del

Centro Regionale di Neurogenetica, Lamezia Terme (Cz).

Iannone F, Crocco P, Dato S, Hoxha E, Montesanto A, Passarino G, Rose G. (2018). "Genetic variability of amino acid transporter genes affects physical decline after the fifth decade of life and human survival". The European Human Genetics Conference (ESHG) in conjunction with the European Meeting on Psychosocial Aspects of Genetics, Milano.

Iannone F, Cione E, Crocco P, Dato S, Montesanto A, Passarino G, Rose G. (2019). "microRNA-133b appears to epigenetically affect the age-related loss of muscle mass". Joint Meeting Associazione Genetica Italiana (AGI) - Società Italiana di Mutagenesi Ambientale e Genomica (SIMAG), Cortona (Ar).

Montesanto A, Geracitano S, Guarasci F, Iannone F, Morelli F, Cosimo SC, Bellizzi D, Rose G, Passarino G. (2019). "Invecchiare in Calabria: dati sulla condizione degli anziani in RSA nelle province di Cosenza e Crotone." Congresso Nazionale Società Italiana di Gerontologia e Geriatria – SIGG – Roma.

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