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Uncoupling to survive: association studies and *in vitro* analyses support the role of UCPs in human longevity

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Sommario

L'invecchiamento è un fenomeno naturale caratterizzato da un progressivo declino della capacità funzionale di mantenere l'omeostasi basale dei vari organi e tessuti e di rispondere adeguatamente, in condizioni di stress, ai bisogni fisiologici. E' noto che il 20% - 30% delle variazione nella durata della vita umana può essere attribuita a fattori genetici, che diventano ancora più rilevanti in età avanzata . Tra i diversi loci genetici e pathways che influenzano questo processo, i mitocondri, essendo i principali siti cellulari che controllano il metabolismo energetico e lo stato redox, occupano un ruolo centrale nella modulazione dell'invecchiamento.

Negli ultimi anni, l'attività disaccoppiante dei mitocondri, ossia il disaccoppiamento della respirazione dalla produzione di energia, è stata considerata come un processo in grado di modulare il tasso di invecchiamento e la durata della vita. Diversi esperimenti in organismi modello hanno dimostrato un ruolo del disaccoppiamento mediato dalle proteine disaccoppianti (UCPs) nell'estensione della durata della vita. Nell'uomo, sono state descritte cinque proteine disaccoppianti (UCP1-5). Queste proteine sembrano funzionare come regolatori dell'omeostasi energetica e come antiossidanti, e, anche se la loro funzione non è stata ancora ben stabilita, è stato suggerito un loro ruolo nell'invecchiamento e nella longevità umana.

Al fine di comprendere il ruolo delle UCPs nell'invecchiamento e nella longevità, durante il mio dottorato di ricerca sono stata coinvolta nello studio degli effetti della variabilità dei geni UCP sulla sopravvivenza in età molto avanzata. Tutte le analisi sono state condotte su un campione reclutato nel Sud Italia.

In primo luogo, ho analizzato la variabilità di due polimorfismi (A-3826G e C-3737A) in forte linkage disequilibrium tra loro e situati al 5 'del gene *UCP1* in una regione che è nota essere coinvolta nell'attivazione trascrizionale del gene. Da questo studio è emerso

che l'aplotipo G-A ha una significativa variazione di frequenza con l'età (p=0,003) e che il diplotipo A-C/A-C ha un aumento molto significativo con l'età (p = 0,005), mentre il diplotipo A-C/G- A subisce una variazione di frequenza legata all'età significativamente negativa (p<0,001). Per verificare se l'attività trascrizionale di questa regione possa essere influenzata dalla variabilità dei due polimorfismi analizzati, è stato anche effettuato uno studio funzionale sia a condizioni basali che dopo stimolazione ormonale. Da questo studio è emerso che il costrutto A-C è up-regolato in seguito a stimolazione con acido retinoico (p=0,027) e con progesterone (p=0,014), mentre è down-regolato (p=0,028) quando le cellule sono trattate con estradiolo. Il costrutto G-A, invece, mostra una bassa upregolazione solo dopo trattamento con acido retinoico (p=0,046). In conclusione, questi risultati suggeriscono che i due polimorfismi analizzati (A-3826G e C-3737) del gene *UCP1* possono modulare la sopravvivenza, probabilmente influenzando i livelli della proteina.

Successivamente, abbiamo studiato due varianti del gene *UCP3*, espresso principalmente nel muscolo scheletrico, e abbiamo valutato se queste varianti fossero correlate a uno dei più importanti marcatori dell'invecchiamento umano, l'Hand Grip Strenght (misurazione strumentale della forza muscolare dell'avambraccio). È emerso che i portatori dell'allele T dell'rs1800849 hanno valori più alti di Hand Grip (p=0.010) e, dal momento che è noto che questo allele aumenta l'espressione del gene, possiamo concludere che un processo di disaccoppiamento più efficiente ha un effetto benefico sull'invecchiamento muscolare causando un rallentamento del suo decadimento correlato all'età.

Infine, allo scopo di analizzare il ruolo di tutti i geni UCP nell'invecchiamento umano, sono stati analizzati dieci polimorfismi dei geni *UCP1-5*. Per valutare gli effetti dei genotipi sulla probabilità di raggiungere età avanzate abbiamo creato un modello di

regressione logistica multivariata. Dall'analisi è emerso che le varianti genetiche *UCP1* (rs12502572-GG), *UCP3* (rs15763-TT e rs1800849-T/-), e *UCP4* (rs9472817-GG) influenzano la possibilità dei maschi di sopravvivere fino ad età molto avanzata (valore di p: p = 0,031, p = 0,017, p = 0,011 ep = 0,008, rispettivamente), mentre nelle femmine solo *UCP3* (rs1800849-T/-) ha mostrato tale associazione (p = 0,016).

Nel complesso, questi risultati suggeriscono che la variabilità genetica dei geni *UCPs* modula la sopravvivenza in età avanzata in maniera sesso-specifica. Dal momento che i geni *UCPs* presentano un'espressione tessuto specifica, i nostri risultati portano a ipotizzare un coinvolgimento del processo di disaccoppiamento nella modulazione della senescenza tessuto-specifica.

Summary

Aging is a natural and complex phenomenon characterized by a progressive decline in the functional capacity of various organs to maintain baseline tissue homeostasis and to respond adequately to physiological needs under stress. It is well known that the 20% - 30% of the variation in human lifespan can be attributed to genetic factors, which become more relevant ad advanced ages. Among several individual loci and pathways affecting this process, mitochondrial function is central to modulation of aging, being mitochondria the main cellular sites controlling energy metabolism and redox state. In recent years, the uncoupling activity of mitochondria, namely the uncoupling of respiration from energy production, has been considered as a process that can modulate the rate of aging and lifespan. Several experiments in model organisms have demonstrated a role for uncoupling mediated by UCP proteins in extending lifespan. In human, five uncoupling proteins (UCP1-5) have been described. These proteins seem to function as regulators of energy homeostasis and as antioxidants, and, even if their effect has not been yet established, a role of UCPs in human aging and longevity has been suggested.

In order to understand the role of UCPs in aging and longevity, during my PhD appointment I was involved in the study of the effects of the variability of UCP genes on survival at very old age. The analyses have been carried out on unrelated southern Italian individuals.

Firstly, we analyzed the variability of two polymorphisms (A-3826G and C-3737A) in strong linkage disequilibrium with each other and located at the 5' of the gene *UCP1* in a region which has been reported to mediate the transcriptional activation of the UCP1 gene. We found that the G-A haplotype showed a significant frequency variation with age ($p=0.003$) and that the diplotype A-C/A-C showed a highly significant increase with

age (p=0.005), while the diplotype A-C/G-A had a significant negative age related frequency variation (p<0.001). To test whether the transcriptional activity of that region was influenced by the haplotype variability, a functional study was also carried out both at basal condition and after stimulation. We found that the A-C constructs was upregulated after retinoic stimulation (p=0.027) and progesterone stimulation (p=0.014), while was down-regulated (p=0.028) when cells were treated with estradiol. The G-A constructs showed a low upregulation only after retinoic stimulation (p= 0.046). In conclusion, these results suggest that the A-3826G and C-3737A of the *UCP1* gene may modulate survival, probably by affecting the levels of the protein.

Subsequently, we studied two variants of the *UCP3* gene mainly expressed in skeletal muscle and we evaluated whether these variants were correlated to hand grip strength, one of the most important landmark of human aging. We found that the carriers of rs1800849 T allele has a significant impact on hand grip strength in our sample (p=0.010). Since this allele has been reported to promote a higher expression of the gene, we conclude that a more efficient uncoupling process has a beneficial effect on the aging muscle by slowing down its age related decay.

Finally, in order to analyze the role of all UCP genes in human aging ten SNPs of UCP1-5 genes were analyzed. To evaluate the effects of UCP genotypes on the probability to reach advanced ages we set up a multivariate logistic-regression model. We found that UCP1(rs12502572-GG), UCP3 (rs15763-TT and rs1800849-T/-), and UCP4 (rs9472817-GG) genetic variation affects male chance to survive to very old ages (p value: p=0.031, p=0.017, p=0.011 and p=0.008, respectively), while in females only the UCP3 (rs1800849-T/-) affects such chance (p=0.016).

On the whole, these results suggest that the UCPs gene variability modulates survival at old ages in a gender-specific way. As UCPs are differently expressed in various organs

and tissues, our results allowed some inferences on the involvement of the uncoupling process (and of energy storage and expenditure) in the elderly.

List of abbrevations

4HNE	4-hydroxy-2 ,3-transnonenale
ADP	Adenosine DiPhosphate
ATP	Adenosine TriPhosphate
BAT	Brown Adipocite Tissue
BMI	Body Mass Index
cDNA	Complementary DeoxyriboNucleic Acid
CoQ	Coenzyme Q
CoQH	Ubisemiquinone
$CoQH_2$	Ubiquinol
CR	Calorie Restriction
CRE	cAMP Response Elements
DNA	DeoxyriboNucleic Acid
e^-	Electron
ETC	Electron Transfer Chain
$FADH_2$	Flavin Adenine DiNucleotide
GDP	Guanosine DiPhosphate
Gly	Glycine
GSH	Glutathione
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen Peroxide
HO_2	Hydroperoxyl
KDa	KiloDalton
Leu	Leucine
Met	Methionine
MREs	MyoD responsive elements
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NADH	Nicotinamide Adenine DiNucleotide
NO	Nitric Oxide
O_2^-	Superoxide anion

OH	Hydroxyl Radical
$ONOO^-$	Oxidant PeroxyNitrite
OXPHOS	Oxidative Phosphorylation
PGC-1α Coactivator	Peroxisome Proliferator-activated receptor- α
Pi	Inorganic Phosphate
PolgA	DNA Polymerase gamma A
PPARs (α and γ)	Peroxisome Proliferator-Activated Receptors
PPRE	Peroxisome Proliferator Response Element
RARE	Retinoic Acid Response Elements
RNAi	RNA interference
ROS	Reactive Oxygen Species
SDH	Succinate DeHydrogenase
Ser	Serine
SNS	Sympathetic Nervous System
SOD	SuperOxide Dismutase
SREBPs	Sterol Regulatory Element Binding Proteins
TRE	Thyroid hormone Responsive Element
UCPs (UCP1,-5)	UnCoupling Proteins (1, 2, 3, 4, 5)
Val	Valine

1. Introduction

Aging is characterized by a progressive decline of the normal physiological functions. It is a complex process that characterizes every biological specie and leads to a dramatic reduction of the individual survival probability and, ultimately, to death. During the aging process continuous changes can be observed not only in the individual anatomy and physiology, but also at cellular and molecular levels. These changes may be characterized by gain, loss or maintenance of structure, function or capability to cope with endogenous and exogenous factors acting as stressors. Aging is a process that affects all organisms, but lifespan is species specific. In addition, a noticeable inter-individual variability exists with respect to the rate and the quality of aging.

Over the past 50 years, in the western society, there has been a gradual increase in the average lifespan of individuals that is mainly due to the improvement of living conditions, environmental hygiene, and to the development and application of new knowledge in medical and pharmaceutical fields (Kannisto, 1994). This has led to an increase in life expectancy which, as evidenced by the mortality curves, sharply increased the relative prevalence of elderly subjects, including nonagenarians and centenarians, in western societies. The increase of lifespan introduces new problems; in fact, the prevalence of age-related diseases such as Parkinson, Alzheimer and heart diseases, have also increased. Since aging-related diseases account for approximately 20% of healthcare costs, there has been a growing scientific interest regarding the study of the aging phenotype and the basis of individual variability in order to better understand which factors affect the quality of aging.

It is well known that environmental conditions (education, socio-economic status, and lifestyle choices such as diet, exercise, smoking habits, etc.) and genetic factors are

essential to modulate human aging and longevity. Several studies have been carried out to separate the genetic contribution from the environmental one. Studies on the variation of lifespan in twins reported that: i) the share of the variation in human life span which can be attributed to genetic differences among individuals ranges between 22% and 33% (McGue et al., 1993; Herskind et al., 1996; Ljungquist et al., 1998); ii) the heritability of longevity is estimated as 0.26 for males and 0.23 for females (Herskind et al., 1996). Subsequently, examples of familial clustering of longevity were reported by Perls and co-workers (Perls et al., 2000). 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 an 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 (age major or equal to 110 years), where a survival advantage was found for siblings and mothers of super-centenarians (Perls et al., 2002). 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. This study clearly supports the existence of a genetic component affecting longevity in humans, especially at advanced ages.

In the last years a large amount of studies aimed at identifying genes involved in aging and longevity in humans (for a review see Fontana et al., 2010). Studies on model organisms showed that the majority of the genetic variants having an influence on lifespan belong to a limited number of pathways, highly conserved during evolution. This suggests that a common "core" of genes and pathways exists, responsible for modulating the lifespan of all animal species. The literature about this argument is huge and concordant to highlight a complex network of interactions linking metabolic pathways for nutrients metabolism and those that regulate response to external and / or internal stress factors. Figure.1 shows this complex network of interactions, whose efficient functioning is supposed to be critical for the modulation of lifespan.

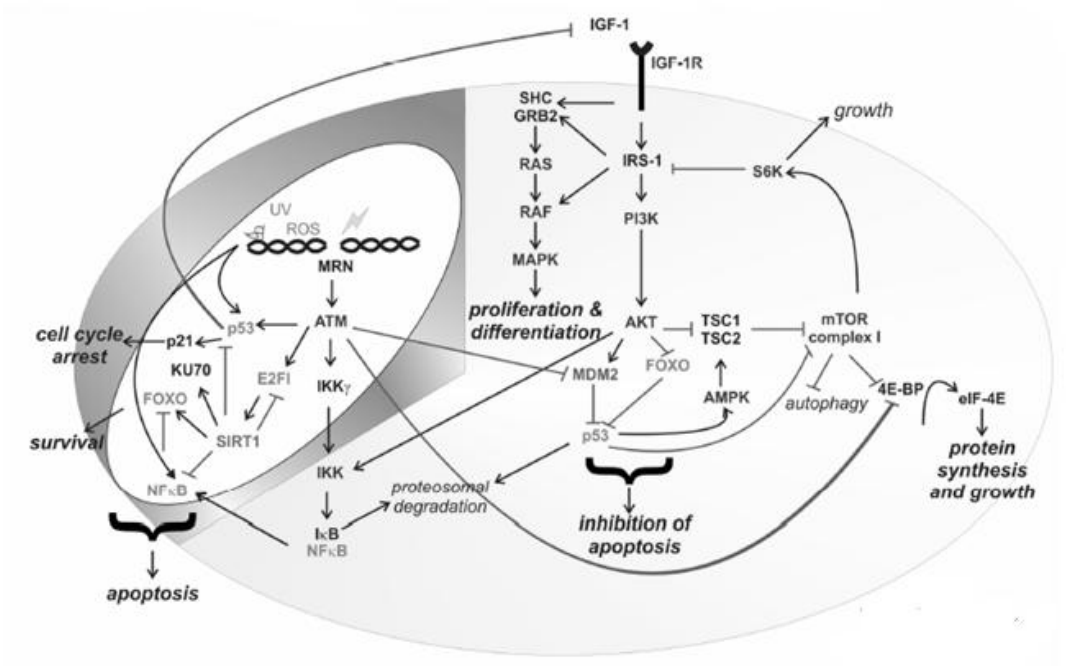

Figure 1*: Network of interaction between metabolism and stress response (from: Niedernhofer and Robbins, 2008).*

In studies on human longevity the model of centenarians has emerged as essential because: i) it personifies the longevity phenotype naturally occurring in an outbreed species; ii) their environment continuously pushes the organism to cope with intrinsic and extrinsic antigenic loads; iii) several changes have been experimented due to the progress observed in the last century in all developed countries across the world; iv) they provide unique insights on the complex network of biological and non biological factors which guide individual survival at old age (De Benedictis and Franceschi, 2006). Taking into account the findings from model organisms, genetic studies in humans forwarded on specific genes belonging to the previously mentionated pathways. Many studies have found different genetic loci affecting longevity: 'cardiovascular genes' (APOE, APOC3, MTTP, ACE), 'immune system genes' (IL6), 'metabolism-related genes' (IGF1, GH1, HFE) and mitochondrial polymorphisms (Christensen et al., 2006 and references therein). As a consequence, due to their biological function, these "longevity genes" may be useful in the study of human aging and longevity. It is important to highlight that human "longevity genes" could function in several important ways: they may slow down the rate of age-related changes in cells and tissues, improve the effectiveness of repair mechanisms, and increase resistance to environmental stresses like infection and injury. Moreover, these genes could also affect a wide spectrum of debilitating age-related conditions.

1.1 Mitochondria: key components of the aging process

Over the last years, due to its crucial role in the energetic balance of the cell, mitochondrion emerged as a key factor in a number of complex traits, including ageing and numerous degenerative diseases. In fact, mitochondria are the organelles where Oxidative Phosphorylation (OXPHOS) takes place, and this implies that mitochondria

have a central position between energy uptake (that is food uptake and metabolism) and energy production. The direct consequence of this process is the mitochondria implication in several cellular processes such as heat production, apoptosis regulation, cellular differentiation, and especially in the production and the regulation of one of the most important by-products of cellular metabolism: the Reactive Oxygen Species (ROS). For both energy and ROS production, mitochondria play a central role in aging.

1.1.1 Mitochondria and energy production

Oxidative phosphorylation involves the coupling of electron transport, through the electron transfer chain (ETC), to the active pumping of protons across the inner mitochondrial membrane and ATP formation by the F1Fo-ATP synthase. The mitochondrial electron transport chain is made up of 80 component proteins that constitute five complexes designated for cellular energy production: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinone cytochrome c oxidoreductase), complex IV (cytochrome oxidase), and complex V (F1Fo-ATP synthase).

The reducing equivalents extracted from the substrates are needed to start the transport of electrons through the mitochondrial ETC. The electron donors, nicotinamide adenine dinucleotide (NADH) and flavin adenine di nucleotide ($FADH_2$), reduce equivalent transferring electrons to the ETC. This transferring is driven by a redox potential that is present across the chain. In particular, NADH is in turn oxidized by complex I (NADH-CoQ reductase), which transfers electrons to membrane-bound electron carrier, the ubiquinone (coenzyme Q, CoQ) to give ubisemiquinone (CoQH.) and then ubiquinol (CoQH2). $FADH_2$ is an alternative substrate for the start of mitochondrial respiration. It is oxidized by complex II (succinate dehydrogenase, SDH), which then transfers

electrons to ubiquinone. The ubiquinol sends electrons to complex III (CoQ cytochrome c reductase), which, in turn, transfers them to cytochrome c oxidized. The reduced cytochrome c passes electrons to complex IV (cytochrome c oxidase), which, in the final step, reduces oxygen to water ($4H^+ + 4e^- + O^2 = 2H_2O$). This process of substrate oxidation and oxygen reduction, is also called "mitochondrial respiration" (Figure 2).

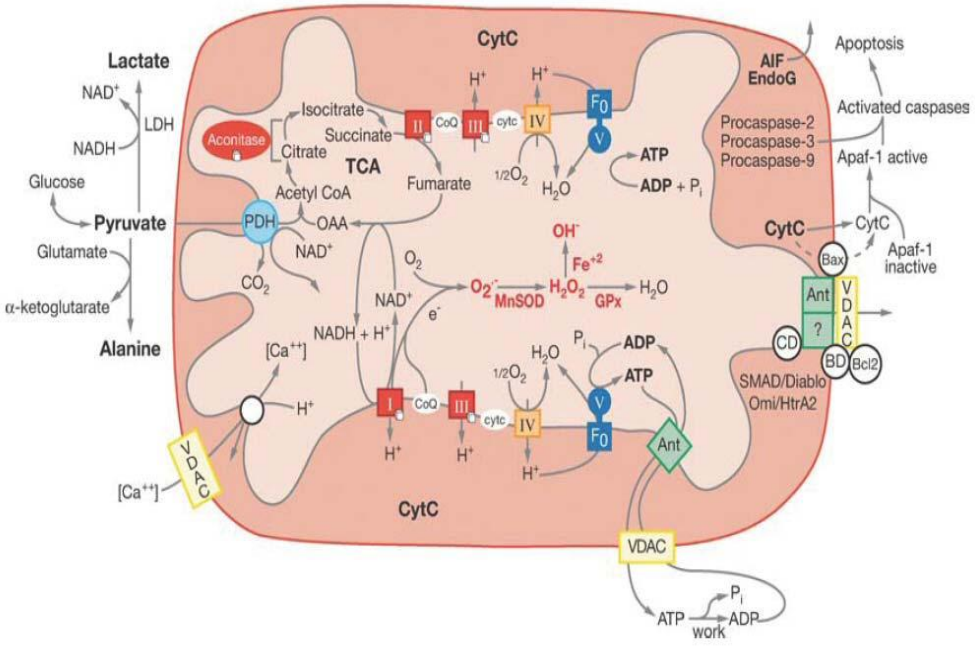

***Figure 2**: Mitochondrial oxidative phosphorylation and ROS production.*

During respiration electron transfer along the redox potential gradient from NADH or $FADH_2$ to oxygen is coupled to the active transport of hydrogen ions from the matrix to the cytosolic side of the inner membrane as described by the chemiosmotic theory of Mitchell (Mitchell, 1976). Complex I, III and IV of the respiratory chain pump protons from the mitochondrial matrix to the mitochondria intermembrane space, thereby establishing a gradient across the inner mitochondrial membrane. This electrochemical proton gradient, ΔP, has two components: a difference in electric potential (ΔΨ) and a difference in proton concentration (ΔpH) across the membrane (ΔP = ΔΨ + ΔpH). The

energy stored in the protons gradient across the inner mitochondrial membrane is used by ATP synthase (complex V) which, when protons are transported from the mitochondrial intermembrane space into the matrix, synthesizes ATP from ADP and inorganic phosphate (Pi) (Wallace DC, 2005). ATP synthase involves two protein complexes, F1 and F0.

Recently, Artal-Sanz and coworkers (2009) demonstrated that a component of the inner membrane of mitochondria (prohibitin) promotes longevity through modulation of mitochondrial proliferation. Moreover, genetic studies in both nematodes and rodents have reported that longevity may be promoted by moderate inactivation of genes important for mitochondrial electron transport chain (ETC) function. Hur and coworkers (2010) performed an RNAi screen in *Drosophila melanogaster* to test the role of ETC components in lifespan modulation. Five ETC genes turned out to be associated with increased longevity. However, only two of the knocked-down ETC genes decrease the abundance of fully assembled respiratory complexes. Moreover, none of the five silenced ETC genes affecting longevity was reported to cause a decrease in ATP levels.

1.1.2 Mitochondria and ROS generation

During oxidative phosphorylation, a small proportion of consumed oxygen, on average 0.4 - 4%, is converted to Reactive Oxigen Species (ROS). ROS includes a variety of molecules and free radicals (chemical species with one unpaired electron) derived from the metabolism of molecular oxygen. These molecules include: Superoxide anion (O_2-), produced by an interaction between an oxygen and an electron escaped from the electron transport chain at other sites; Hydrogen peroxide (H_2O_2), derived from superoxide by a reaction catalized by SOD; Hydroxyl radical (OH-.), one of the most toxic ROS that causes widespread oxidative damage. Moreover, O_2^- may

nonenzymatically react with nitric oxide (NO) to produce the powerful oxidant peroxynitrite ($ONOO^-$) (Beckman et al., 1996; Radi et al., 2002).

Since the diffusion capability of most ROS is limited by their lipid solubility, their effect is mainly exerted on the molecules close to the mitochondrial transport chain where they are produced. These molecules include lipids, proteins and nucleic acids. Lipids can be damaged by free radicals directly by peroxidation or indirectly through the production of highly reactive aldehydes. The 4-hydroxy-2 ,3-transnonenale (4HNE) aldehyde is one of the main products of lipid peroxidation. It causes a variety of harmful effects on the molecules with which it comes into contact. For instance, the interaction between 4HNE and proteins induces structural and functional changes, while the interaction with membrane phospholipids decreases membrane fluidity and permeability. Moreover, such an interaction inhibits metabolic process and alterates ions transport (Nigam et al., 2000). The damage to mitochondria induced by lipid peroxidation can lead to further ROS generation (Green et al., 1998).

As lipids, also proteins are sensitive to ROS. The inner mitochondrial membrane contains a high proportion of protein physically associated with fats. Damage to these proteins, as the direct result of oxidative stress or as a consequence of lipid peroxidation, may occur in abnormal protein aggregation , in their degradation or in their loss of function.

Because of its proximity to the mitochondrial inner membrane and the lack of protective coating provided by histones, mitochondrial DNA (mtDNA) is the primary target of ROS. The 8-hydroxy-2-deoxyguanosine presence, which is the most abundant among the products of nucleotides oxidation, is used as an indicator of oxidative damage against DNA (Chomyn and Attardi, 2003). Several studies have shown that levels of 8-hydroxy-2-deoxyguanosine in mtDNA are higher than those observed in nuclear DNA

(Chung et al, 1992, Agarwal and Sohal, 1994). It has been suggested that oxidative damage is responsible for the accumulation of mutations in mitochondrial genome throughout life. This accumulation leads to synthesis of no functional subunits of the electron transport chain, the production of even more ROS and a consequent further increased mtDNA damage (Hiona et al, 2010).

In the aerobic cells ROS coexist in balance with biochemical antioxidants. Cells have two natural antioxidant systems to restore this balance: enzymes and low molecular weight antioxidants. Enzymatic antioxidant system includes the previously mentioned SOD, catalase, and peroxidase. The low molecular weight antioxidants include ascorbate, glutathione (GSH), phenolic compounds, and tocopherals. When the critical balance between ROS and antioxidants is disrupted (excess of ROS or antioxidants deplation) oxidative stress occurs.

1.1.3 Aging, ROS and the contribution of the uncoupling process

It is generally assumed that accumulate damages to a variety of cellular systems are the underlying cause of aging (Sinclair and Oberdoerffer, 2009). Harman was the first to propose *the free radical theory of aging*. According to this theory aging and age-associated degenerative diseases are attributed to the deleterious effects ROS (specifically hydroxyl, OH-, and hydroperoxyl, HO2-) on various cell components (Harman, 1956). In particular, mitochondria, a major site of ROS production, have a central role in this process (Harman, 1972). Several studies in model organisms confirmed that oxidative damage increases with age and that many forms of ROS may be the cause of accumulated oxidative damage (Bokov et al., 2004). Harman's original hypothesis has been developed and now is commonly known as the oxidative stress theory of aging (Sohal et al., 1996). It is also known that under normal physiological

conditions, a chronic state of oxidative stress exists and this is due to an imbalance between pro- and anti- oxidants (Sohal et al., 1996). This imbalance leads to the accumulation of damages to cellular macromolecules that contribute to a progressive decay of cells and tissues. In this frame, aging process may be directly influenced by the regulation of oxidative stress. Thus lifespan should be increased by a reduction of the oxidative stress, by an increase of the antioxidant defenses or by their combination. Remarkably, in Drosophila the manipulation of mitochondrial antioxidant systems is able to increase lifespan (Addabbo et al., 2009) while in mice this effect is not observed (Perez et al., 2009). To support the oxidative stress theory of aging several studies have analyzed whether long-lived animals have reduced oxidative damage or increased oxidative stress resistance.

In most mammalian models it has been demonstrated that life span can be extended by experimental intervention, such as calorie restriction (CR) or genetic manipulation. "Caloric restriction" means a diet in which calories are reduced by 30-40%, so it is characterized by a reduction of caloric intake without malnutrition. CR is the only non-genetic treatment that clearly increases mean and maximum lifespan in various animal models, including mice and rats (Weindruch and Walford, 1988; Jazwinsk, 2000, Rogina, et al, 2000; Bishop and Guarente, 2007; Sanz and Stefanatos, 2008). This effect on life span seems due to a reduction in oxidative damage/stress (Guarente and Kenyon, 2000). Rodents under CR show reduction in levels of oxidized protein, lipid, and DNA; reduced rates of mitochondrial ROS production, and increased resistance to oxidative stress compared to rodents fed ad libitum (Sohal et al, 1994; Li et al., 1998; Sun et al., 2001; Bokov et al., 2004; Richardson et al., 2004; Harper et al., 2006). However, it must be pointed out that CR alters more than free radical production (e.g. it decreases insulin signaling) and therefore the increase in life span cannot be exclusively attributed

to a decrease in mitochondrial ROS generation (Sanz and Stefanatos, 2008). Similarly, several genetic manipulations extend lifespan causing a reduction in oxidative damage/stress. For instance, a number of studies revealed in animal models where components of complex network of signaling pathways modulated by nutrients (IGF-1, TOR, sirtuins, AMP kinase, and PGC-1α) are up/down regulated phenotype characterized by the slowing of the aging process can be observed (Raffaello and Rizzuto, 2010).

Oxidative damage targeted to mitochondria and mtDNA is supposed to be one of the most important factors in determining age-related cellular decline. The link between mtDNA somatic mutations and aging phenotypes is generally accepted and supported by numerous studies (Kujoth et al. 2005). The "mutator mice" (mice expressing a proofreading-deficient version of the catalytic subunit of mtDNA polymerase, PolgA) are characterized by high levels of mtDNA point mutations and deletions, and display many features of premature aging (Trifunovic et al. 2004; Edgar et al. 2009). However, the link between mtDNA mutations and increased ROS production during age is debated. For instance, in the "mutator mice" the high mutation load in mtDNA was not associated with increased oxidative damage (Trifunovic et al. 2005); on the other hand, mice expressing peroxisomal catalase targeted to mitochondria showed increased lifespan which was associated with decreased damage to mtDNA and increased mitochondrial resistance to ROS damage (Schriner et al. 2005).

A mechanism that could be important for contrasting the accumulation of ROS, in minimizing oxidative damage to DNA, and in slowing aging, is the uncoupling of oxidative phosphorylation from ATP production. During oxidative phosphorylation not all the energy available in the electrochemical gradient is coupled to ATP synthesis.

Reducing the association between substrates oxidation and phosphorylation, decoupling allows to the electron transport chain to proceed without ATP synthesis. This uncoupling activity induces a "proton leak" that is a proton passage from the intermembrane space into the mitochondrial matrix not coupled to the ATP synthase activity. In this process the proton gradient becomes "relaxed", the membrane potential decreases and energy is released as heat (Stuart et al, 1999). This mitochondrial proton cycling, that accounts for up to 20-25% of basal metabolic rate, seems to be a general phenomenon, both in endotherm and ectotherm organisms. The high energetic cost of this futile proton cycle must be offset by high-benefit outcomes. Since it occurs in ectotherms, the heat production cannot be the most important function of the uncoupling process. A possibility is that it is a pathway of energy loss that might have a great importance from an ecological point of view. Interestingly, an important consequence of lowering of the mitochondrial membrane potential is a reduction in ROS generation. This process can justify the high energy costs imposed by the mitochondrial proton leak (Brand, 2000). In fact, even if cells have powerful antioxidant defences to protect themselves against ROS, prevention, rather than cure, would appear to be a more effective way to decrease oxidative damage.

Since ROS-induced damages and energy balance are among the major contributors to the aging process, the “Uncoupling to survive theory" has been proposed. According to this theory, individuals with more coupled mitochondria are subject to less oxidative stress and age slow than animals with fewer uncoupling mitochondria (Brand, 2000; Van Voorhies, 2004; Wolkow and Iser, 2006). A confirmation of this hypothesis comes from a study by Speakman and coworkers (2004).They found that individual mice with a high metabolism lived 36% longer than those with low metabolism. Moreover, they exhibited higher resting oxygen consumption rate and they also possessed more

uncoupled mitochondria. In addition, tightly coupled cells showed greater deterioration with age than relatively uncoupled cells (Amara et al., 2007).

On the whole, these findings support uncoupling as a protective mechanism that minimizes ROS production and preserves mitochondrial function with age.

1.2. Human UnCoupling Proteins (UCPs)

The uncoupling of respiration from ATP production is a mitochondrial process by which stored energy is released as heat. This process is mediated by a group of five mitochondrial transporters present in the mitochondrial inner membrane well-known as UnCoupling Proteins (UCPs) (Krauss et al. 2005). These proteins constitute a subfamily of mitochondrial anion-carriers localized in the inner mitochondrial membrane. As uncouplers, UCPs uncouple ATP synthesis from the respiratory chain by transporting protons into the matrix causing proton motive force dissipation (Yu et al., 2000). UCPs have been identified in different species of invertebrates, including the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* (Hanak and Jezek, 2001; Sokolova and Sokolov, 2005), plants (Laloi et al, 1997), fungi and protozoa (Jarmuszkiewicz et al, 1999). However, the vast majority of UCPs is found in vertebrates including fish (Stuart et al, 1999), birds (Raimbault et al, 2001) and, especially, mammals. The human uncoupling proteins so far identified are five: UCP1, UCP2, UCP3, UCP4 and UCP5.

UCPs have a molecular mass of 31-34 KDa and share a common tripartite structure that consists of three repeat domains (of about 100 aminoacids) each with two hydrophobic regions forming a transmembrane α-helical spanning the mitochondrial inner membrane (Echtay, 2007). The amino- and carboxy-terminal ends protrude in the intermembrane space. In any repeat, the two helices are connected by a long hydrophilic loop that is

oriented on the matrix side of the membrane. It is believed that the functional unit of protein is a homodimer composed of two identical subunits. Site directed mutagenesis experiments suggest that all the α-helices constitute a hydrophilic channel in the UCP core, and that core access is controlled by "gates" formed by the loops (Arechaga et al, 2001).

Early studies have investigated mechanisms that modulate the activity of UCP1. It has been proposed that fatty acids play a role in UCPs positive regulation. Although the fatty acid-mediated activation is not fully understood, two plausible mechanisms have been proposed for the UCP1-mediated proton transport: the proton buffering model and the fatty acid-cycling model (Krauss et al., 2005; Echtay, 2007). The “proton-buffering model” proposed that fatty acids function as cofactor/activator groups for UCP. In this model, fatty acids provide additional carboxyl moiety at the translocation channel through which protons enter the mitochondrial matrix with the help of proton-buffering amino acids. (Klingenberg and Huang, 1999). In the "fatty acid cycling” model, fatty acids, in the anionic form, are transported from the mitochondrial matrix to the intermembrane space through UCP1. Here, they accept protons and, in protonated form, are able to pass through the membrane and reach the matrix where the protons are released and a new cycle can begin (Garlid et al., 1998). The observation of uncoupling mediated by UCP1 in the absence of fatty acids suggests the existence of a proton pathway that operates when the cycle of fatty acids can not take place.

Purine nucleotides (especially ATP and GDP) are believed to regulate the activity of UCPs, but in a negative manner. It has been shown that the addition of purine nucleotides to mitochondria from Brown Adipocite Tissue (BAT) caused a reduction in uncoupled respiration mediated by UCP1 (Rafael et al., 1994). An inhibitory effect by purine nucleotides was also observed for UCP2 and UCP3 (Echtay et al., 2001). On the

basis of the proposed model, the binding of purine nucleotides to the nucleotide binding sites of UCP1 causes a conformational change which is believed to inhibit the transport activity of the protein (Modriansky et al., 1997). By aligning the sequences of the five UCPs, Ivanova et coworkers (2010) found that these binding sites are conserved in all five UCPs, thus reinforcing the idea that purine nucleotides act as UCPs inhibitors (Klingenberg et al., 1999).

By reducing ATP synthesis, and by the attenuation of ROS production, the uncoupling action of UCPs could directly or indirectly influence cellular metabolism. In fact, numerous data have been gathered on how *UCP* gene expression varies in different physiological and pathological processes of great importance (Li et al., 2008; Salopuro et al., 2009; Jia et al., 2010 and references therein). The relevance of the UCPs functions in several interconnected phenotypes is well depicted in Figure 3.

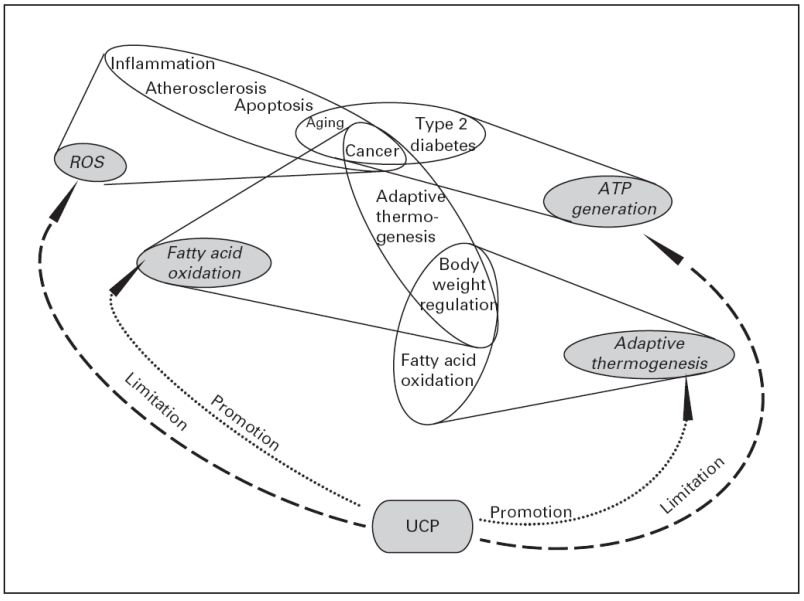

***Figure 3**: Proposed physiological roles and possible implication of uncoupling proteins in pathological events.(from: Nubel and Ricquier 2006).*

The specific role of the five UCPs in the cellular physiology, and how each of them may affect aging and aging-related phenotypes will be discussed in the following sections.

1.2.1 Uncoupling Protein 1 (UCP1)

The Uncoupling Protein1 (UCP1), also known as "thermogenin", was observed for the first time in 1976 in the mitochondria of Brown Adipose Tissue (BAT) (Ricquier and Kader, 1976), and was isolated in 1980 (Lin and Klingenberg, 1980). The cDNA cloning of rat Ucp1 in 1985 (Bouillaud et al, 1985), led to the identification of the amino acid sequence (Bouillaud et al, 1986) of this protein which shows similarity to the ADP / ATP mitochondrial inner membrane carrier (Aquila et al, 1985).

The human *UCP1* gene is located on the long arm of chromosome 4 (4q28.31) (Cassard-Doulcier et al, 1990). This gene is 13 kb long and has a structure highly conserved in rats and mice (where *Ucp1* is located on chromosome 19 and 8, respectively). It contains six exons, each of which encodes for a transmembrane domain.

The regulation of *UCP1* gene occurs mainly at the transcriptional level. It has been well described in murine model, although major features of the transcriptional regulation of the mouse and human *UCP1* genes appear to be similar (Sears et al., 1996; del Mar Gonzalez-Barroso et al., 2000).

A complex enhancer region exists at -3,500 in humans (around -2,500 in rodents) . This region is a multipartite response element with many response elements within a short sequence. cAMP response elements (CRE), retinoic acid response elements (RARE), containing three pairs of half-sites for RXR and RAR, are found which are functional.

PPAR response elements are also found (PPRE); both PPARα and PPARγ can bind these elements. Responsiveness to thyroid hormone (TRE) is also located here. These sites amplify the effect of norepinephrine on *UCP1* transcription (del Mar Gonzalez-Barroso, et al, 2000; Cannon and Nedergaard, 2004 and references therein).

Moreover, upregulation of *UCP1* expression in adipocytes is possible by chromatin remodeling. It has been proposed that gene silencing mechanisms involving DNA methylation of CRE motif, which contains a CpG dinucleotide, may be important in regulating *UCP1* expression (Mancini et al., 1998; Kroft et al., 2001; Demura and Bulun, 2008). Shore and coworkers (Shore et al., 2010) demonstrated that in the murine *Ucp1* enhancer the methylation state of CpG dinucleotides occurs at specific position and shows adipose tissue-specific patterns.

The role of UCP1 in adaptive thermogenesis is well established (Argyropoulos and Harper, 2002). Thermogenesis is a process that allows endotherm animals (mammals and birds) to produce heat. In some circumstances, such as exposure to cold, animals need to produce much more heat. As stated before, rather than using ATP to generate futile work as shivering, a way to achieve heat is to completely bypass the ATP generation system and to allow protons to turn back into the mitochondrial matrix where they can freely react with O_2 forming water and releasing their energy directly as heat (Speakman, 2003) (Figure 4).

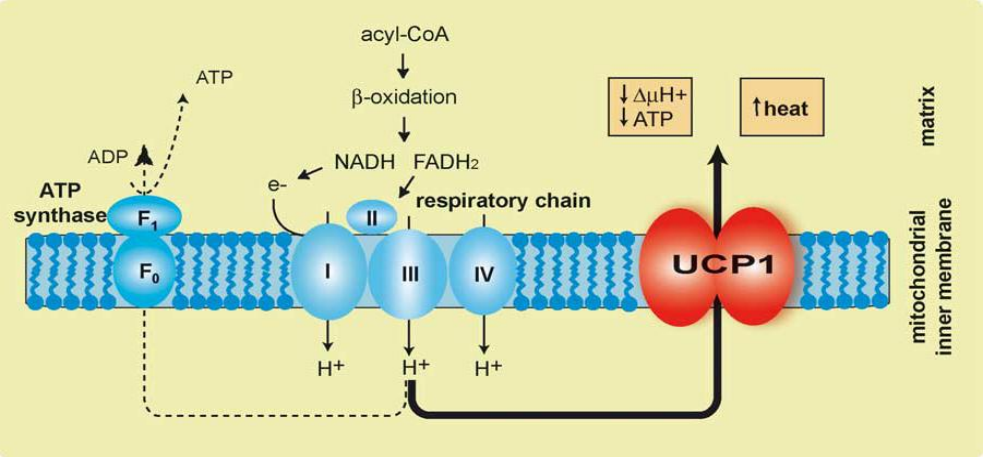

Figure 4: *UCP1 location into inner mitochondrial membrane where is involved in heat production by dissipating the proton gradient.*

Non-shivering thermogenesis, as opposed to shivering thermogenesis that occurs in skeletal muscle, occurs in BAT of mammals, which is specialized in this form of thermogenesis. BAT is located in the perirenal and interscapular areas of rodents, hibernating animals and, as will subsequently be discussed, in human (Mozo, et al, 2005). A high vascularization of BAT allows heat transfer to the tissues perfused by the blood that passes through the BAT (Smith, 1964). Cold exposure increases BAT vascularization by a mechanism involving stimulation of angiogenesis by SNS activation (Asano et al., 1997).

A rapid and full uncoupling of respiration from ATP synthesis leading to thermogenesis requires a large number of UCP1 molecules (Mattson et al., 2010). As described in figure 5, the expression of the *UCP1* gene is directly regulated by peroxisome proliferator-activated receptors (PPARs) in association with adipogenic differentiation (via PPARγ) and in coordination with induction of gene expression required for active thermogenesis and fatty acid oxidation (via PPARα).

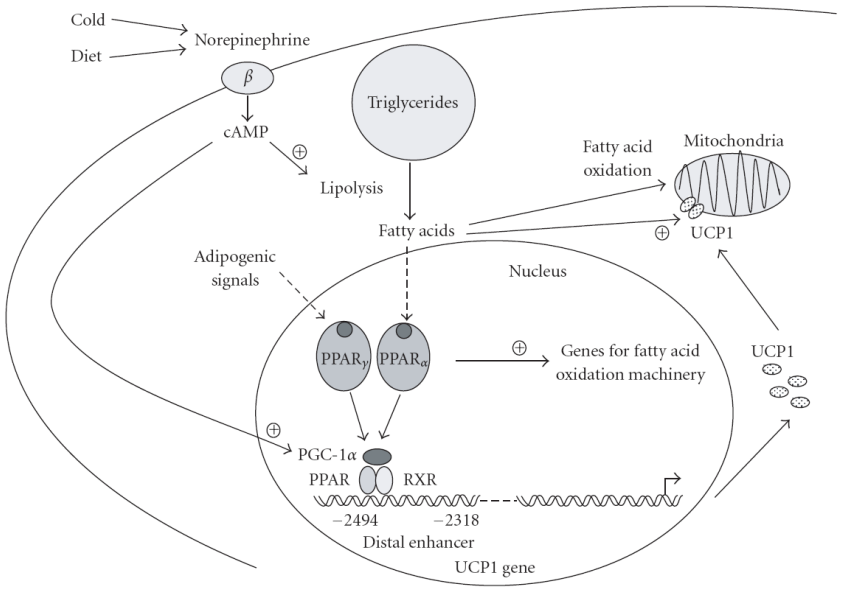

Figure 5*: Schematic representation of the regulation of UCP1 gene expression (from: Villaroya et al., 2007)*

Therefore, in response to certain stimuli such as cold or diet there is a norepinephrine release from the sympathetic nervous system innervating BAT. This norepinephrine release, by acting through β-adrenergic, cAMPdependent pathways and causing the activation of PPARα , PPARγ , and PGC-1α, contributes to the coordination of *UCP1* gene transcription (Sell et al, 2004; Villaroya et al., 2007).

If the primary role of this protein in inducing thermogenesis is well recognized, recently it has been shown that an *in vitro UCP1* overexpression adenovirus-mediated protects neurons from glucose induced degeneration by preventing mitochondrial hyperpolarization and ROS formation. That suggests a possible role of UCP1 in attenuation of ROS production (Echtay et al 2002; Vincent et al, 2004; Wolkow and Iser, 2006;).

Studies on model organisms have unequivocally demonstrated that BAT and UCP1 activity play an essential role in energy balance and in body weight control.

Experimental studies carried out on animals lacking of BAT or UCP1 showed that BAT thermogenesis protects against diet-induced obesity. For example, mice lacking UCP1 exhibit a marked attenuation of SNS-induced fatty acid utilization and nonshivering thermogenesis, which leads to obesity when the mice are fed a high fat diet (Kontani et al., 2005). Moreover, this study showed that, under a normal diet and at usual warm housing temperature, UCP1 deficient mice not develop obesity and appear to live a normal lifespan. In another study it has been shown that eliminating BAT noradrenergic input by disruption of the dopamine β-hydroxylase gene, mice were hyperphagic and more sensible to cold temperatures, but did not become obese because their basal metabolic rate was elevated (Thomas and Palmiter, 1997). Interestingly, in rodents the decline of the UCP1-mediated thermogenesis during aging contribute to weight gain and visceral adiposity, two phenomena that are involved in the development of age-related conditions (McDonald and Horwitz, 1999).

For a long time, it was believed that human thermogenin was expressed only in the newborn, however, recent studies have shown that UCP1 expression can be induced in adulthood. In fact, it has been found that human adults have several discrete areas of functional, UCP1-expressing brown cells and that in response to certain stimuli such as cold exposure and sympathetic stimulation, the white adipocytes can acquire features of brown cells (Tiraby et al., 2007; Nedergaard, 2007; Zingaretti et al., 2009). Moreover, although *UCP1* is mainly expressed in BAT, recent works have shown that *UCP1* is also expressed, although at low levels, in islet cells (Sale et al., 2007), and in thymocytes (Adams et al., 2008). Altogether, these findings strongly suggest that BAT likely plays a role in the regulation of body weight in human.

Population studies were carried out to elucidate the role of the *UCP1* gene variability in diabetes mellitus, obesity, and related metabolic disorders (Jia et al, 2009). The

polymorphisms A-1766G and A-112C at the 5'-flanking region and Ala64Thr polymorphism in exon 2 of *UCP1* gene were studied in the Caucasian and Eastern Asian population (Japanese and Korean) where they are associated with body fat accumulation and body weight gain or body mass index (BMI) (Hamann et al., 1998; Herrmann et al., 2003; Kim et al., 2005; Kim et al., 2006). It has been shown that mutations, changing the activity or gene expression, change the uncoupling activity and impact pancreatic functions and insulin secretion. Two polymorphyisms in linkage disequilibrium, an A to C transition in exon 1 and a Met229Leu substitution in exon 5, were both associated with susceptibility to type-2 diabetes (Mori, 2001). The A-3826G transition is the *UCP1* polymorphism most extensively studied. It is located at the 5' of the gene in a region important for the gene regulation. Interestingly, this polymorphism was found to be associated with reduced mRNA expression indicating that the polymorphism has a functional significance (Sramkova et al, 2007). It is in strong linkage disequilibrium with a C to A variation at -3737, which affects a consensus site for the binding of members of the ATF/CREB (Activing Transcription Factors/cAMP Response Element Binding) family of transcription factors (Rousset et al., 2002). The A-3826G polymorphism has been extensively studied in relation to obesity phenotypes, diabetes mellitus and lipid/lipoprotein-related disease but the results were controversial (Jia et al, 2009 and reference therein).

Taken together, all of these data indicate that the *UCP1* gene is an excellent candidate for these diseases even if further studies are required to investigate genetic polymorphisms of *UCP1* in various populations to better elucidate the molecular and metabolic mechanism of association of these polymorphisms with obesity phenotypes, diabetes mellitus and lipid/lipoprotein-related disease.

1.2.2 Uncoupling Protein 2 (UCP2) and 3 (UCP3)

Human *UCP2* and *UCP3* genes form a cluster on chromosome 11 (11q13). *UCP2* and *UCP3* are very similar to each other, about 70% of homology, and have more than 50% of homology with *UCP1* (59% and 57% respectively). Human *UCP2* contains 8 exons and is 8 Kb long (Fleury et al, 1997). Exons 1 and 2 are not translated and the promoter region does not have a TATA box or CAAT box, although it contains a region rich in GC absent in *UCP1*. A particular feature of human and mouse *Ucp2* gene is the presence of different ATGs in frame with an open reading frame for an unknown peptide of 36 amino acid in exon 2, while the *UCP2* coding sequences starts in exon 3 (Pecqueur et al, 1999). *UCP2* is ubiquitously expressed in different tissues including neurons (Fleury et al., 1997).

UCP3 human gene is located at 7 kb upstream of *UCP2* (Pecqueur et al, 1999). It contains seven exons and is 8.5 Kb long. *UCP3* cDNA cloning revealed that the human gene is expressed as two variants generated by alternative splicing in the last intron. The amino acid sequences correspond to a protein of 312 amino acids, the long-form UCP-3L, and one of 275-amino acids, the short-form UCP-3S. UCP3S contains 5 putative transmembrane domains, while UCP3L contains an additional 37 amino acids at its C terminus that encodes a putative transmembrane domain and a putative purine nucleotide-binding domain. Human *UCP3* is mainly expressed in the skeletal and heart muscle (Boss et al., 1997).

UCP2 and UCP3 discovery in mitochondria of various mammalian tissues, and evidence of their homology with UCP1 initially suggested that these two proteins were also involved in thermogenesis and regulation of energy expenditure (Boss et al, 1997). However, compared to UCP1, UCP2 and -3 are present in very low concentrations and they transport protons only when specifically activated (Esteves and Brand, 2005). This

observation, together with UCPs discovery in ectothermic fish and plants that do not require thermogenesis led to consider the possibility that uncoupling mediated by these proteins has a different and more general function. It has been observed that UCP2 and UCP3 activation causes a "mild uncoupling" which induces a limited increment in proton conductance, so that protonmotrice force is only slightly lowered, the respiration rate increased slightly, and the ATP synthesis can still occurs. This “mild uncoupling“ is sufficient to ensure a strong reduction of mitochondrial ROS production (Brand et al, 2004). Therefore, as UCP1, UCP2 and UCP3 are supposed to be involved in the control of reactive oxygen species production (Krauss et al., 2005). Numerous experimental evidences support this role. *UCP*-knockout mice have increased levels of ROS and showed signs of increased oxidative damage (Argyropoulos and Harper 2002; Rousset, et al., 2004; Echtay, 2007). For instance *UCP3* knockout mice showed a higher ROS production (Brand et al, 2002), while *UCP2* knockout mice were more resistant to parasitic infections, due to increased ROS production in their macrophages (Arsenijevic, et al, 2000). Furthermore, inhibition of UCP2 and 3 mediated by purine nucleotides increases the membrane potential and mitochondrial ROS production (Brand and Esteves, 2005).

It has been observed that UCP2 and UCP3 catalyze an inducible proton conductance in presence of specific activators, such as the aldehyde 4-hydroxynonenal (4HNE) and other aldehydes responsive. Proton conductance in the presence of these activators is inhibited, as previously reported, by ATP and GDP and is favored by fatty acids, which most likely act by removing inhibition induced by purine nucleotides (Rial et al, 2004). In addition, fatty acids increase *UCP2* and *UCP3* genes expression suggesting that proteins encoded by these genes are somehow involved in fatty acids metabolism. The mechanisms by which exposure to fatty acids increases *UCP2* and *UCP3* expression

have not been fully characterized. In addition to sterol regulatory element binding proteins (SREBPs), the G protein-coupled receptor GPR40, selectively expressed in *β*-cells and activated by fatty acids, seems to be implicated in *UCP2* regulation (Villaroya et al., 2007 and references therein). Several studies have also evidenced that *UCP2* and *UCP3* are under the control of PPARs, nuclear hormone receptors acting as sensor for fatty acids and cholesterol-derived metabolites (Wang, 2010). It has been found that the proximal region of the *UCP3* promoter contains PPAR responsive element and uncanonical MyoD responsive elements (MREs) which interact with critical elements in the 3′part of intron 1 to obtain a full response to fasting (Girousse et al., 2009) (Figure 6).

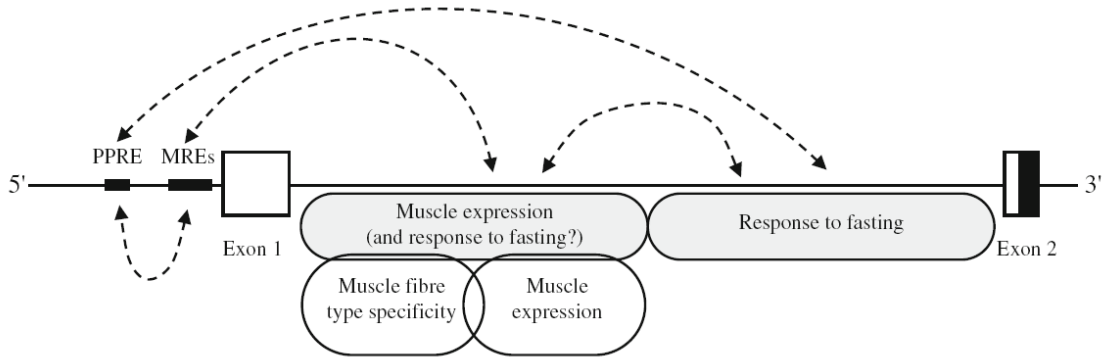

Figure 6*: Potential interactions between cis-acting elements in the promoter and different regions of intron 1 of the human UCP3 gene. MREs, uncanonical MyoD responsive elements; PPRE, PPAR responsive element (from: Girousse et al, 2009).*

Moreover, the regulation of these uncoupling proteins by dietary alterations, thyroid hormones and agonists of the β-3 adrenergic receptor, supports the hypothesis that UCP2 and UCP3 could play an important role in energy balance and body weight regulation (Bezaire et al., 2005; Echtay, 2007; Jia et al., 2009). In mice, high-fat feeding led to up-regulation of *UCP2* in white adipose tissue, brown adipose tissue and skeletal

muscle. Likewise, *UCP3* expression is elevated during states that are associated with increased fat metabolism in rodents and humans, for example, fasting, acute exercise, and high-fat feeding (Echtay, 2007).

Beside these roles, a tissue-specific function for UCP2 and UCP3 it has been also proposed. As for UCP2, implication in insulin secretion from β-cells and in the neuroprotection have been documented. Glucose uptake contributes to mitochondrial respiration for the production of ATP from ADP. The increase in ATP concentrations allows insulin to be released into the bloodstream. When the ATP/ADP ratios is high, *UCP2* may be activated. This activation cause an attenuation of ATP production reducing the rate of insulin vesicle fusion, decreasing insulin release, and attenuating glucose uptake (Chan et al., 2001). Experiments in model systems, that either over- or under-express *UCP2* gene, showed that *UCP2* down-regulate the ability of beta cells to secret insulin. Moreover, induction of Ucp2 deficiency in ob/ob mice partially slow down the development of diabetes and insulin resistance (Zhang et al., 2001). This is of particular interest in metabolic control of complex phenotypes such as type 2 diabetes and aging.

It has been also showed that UCP2 affects several mechanisms involved in neuronal cell death, including excitotoxicity, mitochondria-mediated cell death and ROS. (Mattiasson and Sullivan, 2006). For these reasons UCP2 induction was proposed to have a potential therapeutic effect in the treatment of age-related neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, brain hypoxia and stroke.

As for *UCP3*, the high expression of *UCP3* in skeletal muscle suggested that the gene could be important in the regulation of energy metabolism in this organ. Several observations support the proposed function of UCP3 as fatty acid anion transporter for increasing fatty acid oxidation capacity. Muscle UCP3 protein levels are increased

when rats are fed a diet high in long-chain triglycerides but not a diet high in medium-chain triglycerides which are oxidized via a different pathway (Schrauwen et al., 2003). Skeletal muscle mitochondria of mice overexpression *Ucp3* show increased fatty acid oxidation rates and decreased intramuscular triglyceride stores (Wang et al., 2003).
Nabben and coworkers (2008) showed that the physiological consequence of *UCP3* overexpression in skeletal muscle might be the slowing down in the decline in muscle performance with ageing as a result of a decreased production of ROS, an increased protection of mitochondria from lipid peroxidation, and a better metabolic efficiency.

The variability of *UCP2* and *UCP3* genes has been analyzed in relation to different complex traits. Several polymorphisms in human *UCP2* gene have been identified. In particular, the missense Ala55Val SNP (exon 5) and the G-866A polymorphism in the promoter region (Jia et al., 2009) are the more extensively studied. The Ala55Val polymorphism of the *UCP2* gene it has been fully investigated. It has been shown that VV genotype causes a lower degree of uncoupling, lower energy expenditure (Astrup et al., 1999), higher exercise energy efficiency (Buemann et al 2001), and higher metabolic rate and risk in obesity, as well as a higher incidence of diabetes (Walder et al., 1998; Yu et al., 2005). Moreover, people with the VV genotype had greater weight loss and a higher BMI (Chen et al., 2007). Nevertheless, other studies indicated no association between this polymorphism and metabolic phenotypic features (Dalgaard et al., 2003; Wang et al., 2004; Hsu et al., 2008; Lee et al., 2008). Sesti and coworkers (2003) have noted that the G-866A polymorphism is associated with reduced insulin secretion with a "dose effect" of the A. The G-866A polymorphism was also identified as a risk factor in the development of multiple sclerosis, a chronic inflammatory disease caused by an autoimmune response directed against the myelin sheaths of the nerve

fibers. Also in this case, it has been hypothesized a "dose effect " for the A allele: carriers of homozygote genotype for the mutant allele are more protected against disease than heterozygote (Otaegui et al, 2007).

Genetic variants were also identified in the *UCP3* gene. In particular, the best characterized polymorphism is the C-55T promoter region polymorphism. This polymorphism was associated with a significant increased gene expression in skeletal muscle (Schrauwen et al., 1999). It was also observed a positive correlation between resting metabolic rate and *UCP3* expression. A low resting metabolic rate is a factor that predisposes to weight gain, therefore, higher UCP3 levels could cause a high resting metabolic rate and thus less prone to weight gain (Schrauwen et al, 1999).

To improve the statistical power in association studies and to better verify the role of A-866G and the 45 bp insertion of *UCP2* gene and the C–55T variant of *UCP3* gene (region expanding for a small region of 40 kb), a haplotype study was performed in relation to obesity and type 2 diabetes. It has been reported (Esterbauer et al., 2001) that haplotypes that included the 866A-allele and the 45 bp insertion were more frequent in lean compared with obese subjects and tend to be a protecting factor against T2D (Liu et al., 2005). Moreover, subjects carrying the two variants have been found to have a higher risk of type 2 diabetes (Wang et al., 2004). Even if in some studies (Ochoa et al., 2007) the individual polymorphisms are not found associated with obesity, the haplotype (–866G, De/45 bp, –55T) is associated significantly with obesity and causes a ninefold increase in the risk of the insulin resistance. Thus, the haplotype (–866A, Ins/45 bp, –55C) may protect against insulin resistance in obese populations.

1.2.3 Uncoupling Protein 4 (UCP4) and 5 (UCP5)

UCP4 (also called *SLC25A27*) and *UCP5* (also termed brain-specific mitochondrial carrier protein-1 (*BMCP1*) or *SLC25A14*) are located on chromosomes 6 (6p12.3) and X (Xp24), respectively. *UCP4* and *UCP5* have about 30% of homology with the other members of UCP family (Adams et al., 2000; Graier et al., 2008). Human *UCP4* gene contains 9 exons and 8 introns and is 24,3 Kb long, while *UCP5* gene contains 6 exons and 5 introns and is 33,29 Kb long.

These two proteins, together with UCP2, are known as neuronal UCPs because are widespread in the brain. *UCP4* is mainly expressed in central nervous system and at a lower level in other tissues (Yang et al., 2002; Krauss et al, 2005; Smorodchenko et al., 2009). *UCP5* is particularly expressed in the brain and testis, and is also widely present at lower levels in other tissues such as kidney, uterus, heart, lung, stomach, liver, and skeletal muscle (Sanchis et al., 1998; Andrews et al., 2005; Echtay, 2007). UCP5 has three isoforms: a "long" form of 325-amino acid (UCP5L), a "short" form of 322-amino acid (UCP5S) that lacks amino acids Val–Ser–Gly (VSG) at position 23–25 of UCP5L, and a "short insert" form of 353-amino acid (UCP5SI) that lacks the VSG amino acids but has a 31-amino acid insertion between transmembrane domains III and IV (Yu et al., 2000). The presence of multiple isoforms with a tissue-specific expression suggests a complexity in the *UCP5* regulation.

Even if *UCP4* and *UCP5* are more widespread expressed in the brain than *UCP2*, their functions in neurons have been not completely established. Neurons are characterized by a very high metabolic rate and consequently by a high production of ROS, therefore, it has been presumed that by uncoupling activity, UCP4 and UCP5 may modulate oxidative metabolism in these cells. Andrews and co-workers observed that neuronal uncoupling activity leads to a decreased ROS levels, a decreased Ca^{2+} voltage-

dependent influx and increased local temperature in neuronal microenvironment (Andrews et al, 2005). It has been shown that UCP4 activity can also induces an adaptative shift in energy metabolism, from mitochondrial respiration to glycolysis, that helps sustain neurons under conditions of metabolic and oxidative stress (Liu et al., 2006). Therefore, by decreasing free radical production and stabilizing cellular calcium homeostasis, *UCPs* expressed in neurons may positively influence neuronal function (synaptic transmission and plasticity) and retard cellular deterioration associated, for instance, with neurological disorders. It has been observed that increased UCP2, UCP4, and UCP5 levels (Sullivan et al., 2004), induced by dietary restriction and 2-deoxyglucose administration, improve behavioral outcomes and reduce dopaminergic neurodegeneration in models of Parkinson's disease (Duan and Mattson, 1999). Experimental evidences also showed that nutritional and temperature manipulations are able to modulated the mRNA abundance of *UCP4* and *UCP5* in a tissue-specific manner, suggesting their involvement in metabolic rate and adaptative thermoregulation (Yu et al., 2000; Yang et al., 2002; Andrews et al., 2005). For instance, their uncoupling activity may provide a basis for temperature as a neuromodulator. Moreover, temperature is critical for normal spermatogenesis (Steinberger, 1991), and it is conceivable that UCP5 may be involved in the regulation of local testicular temperature. The function of UCP4 in adipocytes is already unknown.

UCP4 and UCP5 are less studied with respect to UCP1, UCP2, and UCP3 and less is known about their regulation. A study carried out on bovine mammary epithelial cells (bMEC) showed that physiological concentrations of saturated fatty acids (stearate and palmitate), but not unsaturated fatty acids (oleate and linoleate), induced an elevated expression of *UCP5*, but not *UCP4*. Moreover, treatment with insulin induced down-regulation of *UCP4* and *UCP5*. These results suggest that UCP4 and UCP5 are

regulated by insulin and/or fatty acids in mammary epithelial cells and lactating mammary glands, and thereby may play an important role in lipid and energy metabolism (Yonezawa et al., 2009).

Recently, Ho and coworkers (2010) characterized several cis-acting elements that might regulate *UCP4* expression. They found that core promoter activity exists within 100 bp upstream of the transcription initiation site. In *UCP4* transcription a CAAT box (-33/-27) and Sp1 (-62/-49) elements act synergistically. Moreover, a NF-kappaB putative binding site at -507/-495 exists, and it has been observed that mutation of this site decreases significantly the activity of the promoter. Activation of NF-kappaB by TNFalpha or cycloheximide increased, whereas its inhibition by 4-hydroxy-2-nonenal or transfection of pIkBαM suppressed, *UCP4* promoter activity.

UCP4 and *UCP5* variability has been analyzed in relation to several complex phenotypes such as multiple sclerosis, leukoaraiosis, and schizophrenia. It was found that CC genotype for rs10807344 of *UCP4* gene exerts a protective effect on occurrence of multiple sclerosis and of leukoaraiosis that is a vascular demyelinization of the white matter of the brain (Szolnoki et al, 2009 and 2010).

In a case-control study tag-SNPs for the neuronal *UCPs* were investigated in relation to schizophrenia. Modest associations was found for rs10807344 and rs2270450 in *UCP4*. Interestingly, a statistically significant synergistic interaction between *UCP2* and *UCP4* was found, suggesting that *UCP2* and *UCP4* have a modest but important involvement in the genetic etiology of schizophrenia (Yasuno et al., 2007).

Taken together, these data suggest that UCP4 and UCP5 play a role in antioxidant protection and preservation of neuronal dysfunction and for this reasons maybe

implicated in neuroprotection against mitochondrial dysfunction in various degenerative diseases.

1.3. The role of uncoupling proteins in lifespan

Based on the different functions of the uncoupling proteins described in previous sections, uncoupling proteins may provide a link between mitochondria, metabolism and lifespan. In fact, ROS production, metabolic rate and homeostasis are interconnected factors that are all likely to contribute to the accumulation of damage and dysfunction observed during life.

For this reason, starting from "The uncoupling theory of aging" researchers try to highlight the connection between UCP-mediated uncoupling and lifespan (for reviews see: Harper et al., 2004; Wolkow and Iser, 2006; Dietrich and Horvath, 2010). The first evidence of the direct effect of UCPs in senescence came in 2005 by Fridell and coworkers (2005). In their work the authors overespressed the human *UCP2* in adult fly neurons, and found an extantion of lispan. In these transgenic flies, they found an increase in proton leak, and a decrease in ROS production and oxidative damage. The same authors demonstrated that transgenic *Drosophila* lines which targeted *UCP* expression in insulin producing cells showed an attenuated systemic insulin signaling and a significant lifespan extantion (Fridell et al., 2009). In another study, Conti and coworkers (2006) generated transgenic mouse over-expressing *UCP2* in neurons. They found that these mice have increased lifespan. Additional data on the effect of UCP2 regard to mammalian longevity came from the study of Andrews and Horvath (2009) who showed that knockout mice had a significantly shorter survival age compared to their wild-type littermates. However these results show some contradictions with respect to the study by McDonald and coworkers (2008). Interestingly recent

observations have shown that mitochondrial uncoupling mimics metabolic and lifespan effects of caloric restriction. In fact, it has been found that induced mitochondrial mild uncoupling induced the beneficial effects of caloric restriction by reducing oxidative stress, body weight, serum glucose and triglyceride levels (Figure 7).

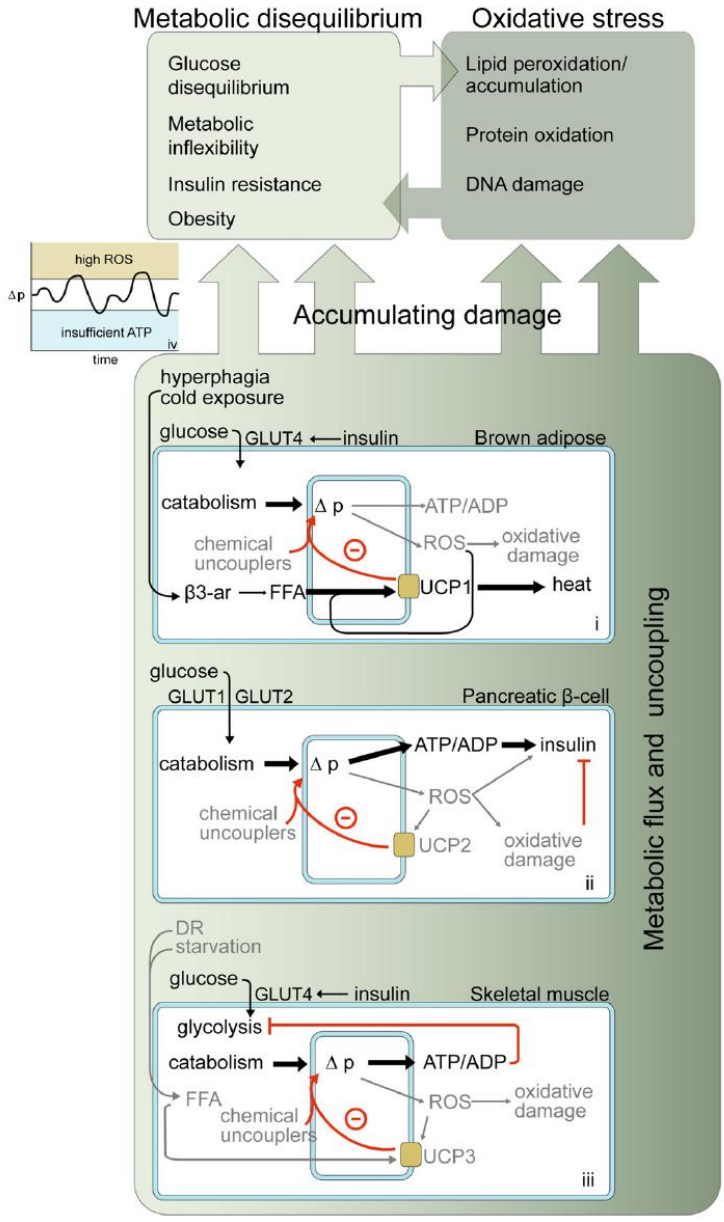

Figure 7*: Different metabolic systems in which mitochondrial uncoupling may function (from: Mookerjee et al., 2010).*

More importantly, mild uncoupling significantly increased lifespan (Caldeira da Silva et al., 2008).Consistently, mice subjected to caloric restriction show an increased expression of both mRNA and protein levels of UCP2 and UCP3 (Bevilacqua et al., 2005; McDonald et al., 2008).

1.4. Plan of the thesis

During my PhD appointment I was involved in the study of the variability of *UCP* genes in relation to aging and longevity. In the following chapters the results of these investigations are reported. In particular, the first part reports the results of a study focused on a genetic and functional investigation of the *UCP1* promoter region. This investigation are described in a manuscript titled "Two variants of the upstream enhancer of human *UCP1* gene affect the expression of the gene and are correlated with human longevity" and has been submitted to Experimental Gerontology. The second part reports the correlation between *UCP3* gene variability and Hand Grip Stregth. This exploration is described in a manuscript titled "A common UCP3 promoter polymorphism influences hand grip strength in elderly people", which is submitted to Biogerontology. Finally, the third part reports the manuscript entitled "Further support to the Uncoupling-to-Survive theory: the genetic variation of human UCP genes is associated with longevity", which will be submitted to Mechanisms of Aging and Development.

2. Two variants of the upstream enhancer of human UCP1 gene affect the expression of the gene and are correlated with human longevity

(Submitted to Experimental Gerontology)

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Abstract

The brown fat specific uncoupling protein 1 (UCP1) is involved in thermogenesis, a process by which energy is dissipated as heat in response to cold stress and excess caloric intake. Thermogenesis has potential implications for body mass control and cellular fat metabolism. In fact, in humans, the UCP1 variability in the 5' region of the *UCP1* gene is associated with obesity, fat gain and metabolism. Since fat metabolism is one of the key-pathways in lifespan extension, we tested the possible effects of this polymorphism on survival.

Two polymorphisms (A-3826G and C-3740A) falling in the promoter region of *UCP1* were analyzed in a sample of 682 subjects from southern Italy (363 women and 319 men; age range 40-109). To test for the functional role of these polymorphisms we cloned in pGL3 promoter vector upstream of the Luc-transcriptional unit the sequences containing the different possible haplotypes. By analysing haplotype specific survival functions we found that the A-C halpotype favours survival in the elderly. Consistently, transfection experiments showed that the luciferase activity of the construct containing the A-C haplotype was significantly higher than that containing G-A haplotype. The results we present suggest a correlation between the activity of UCP1 and human survival, indicating once again the intricacy of mechanisms of energy production, storage and consumption as a key to understand human aging and human longevity.

Key words: Uncoupling proteins; thermogenesis; energy storage; longevity.

1. Introduction

The UnCoupling Protein 1 (UCP1), also known as thermogenin**,** belongs to a family of anion transporters located in the inner membrane of mitochondria. By allowing the leak of protons through the inner mitochondrial membrane of brown adipose tissue (BAT), UCP1 uncouples fuel oxidation from ATP synthesis. This function of UCP1 is mediated by the sympathetic nervous system through noradrenaline release, which activates the β-adrenergic receptors and a cascade of events leading to thermogenesis a process by which energy is dissipated as heat in response to cold stress and excess caloric intake (Cannon and Nedergard, 2004). There is a huge body of evidence that UCP1-mediated thermogenesis and brown fat in rodents can have regulatory effects on body weight, energy balance, glucose and lipid metabolism (ell et al., 2004). A possible role for thermogenin in mitigating the production of oxygen free radicals has also been raised, although available data are still not conclusive on this point (Cannon et al., 2006; Oelkrug et al., 2010). In human, *UCP1* has been thought to be exclusively expressed in BAT which is well developed only in newborns and young children; however, some recent works have shown that the *UCP1* is also expressed, although at low levels, in islet cells (Sale et al., 2007), and in thymocytes (Adams et al., 2008). Moreover, it has been found that human adults have several discrete areas of functional, UCP1-expressing brown cells (Nedergaard et al., 2007, Zingaretti et al., 2009), and that in response to certain stimuli as cold exposure and sympathetic stimulation human white adipocytes can acquire features of brown cells (Tiraby et al., 2003). These findings suggest that also in human adults the processes of energy expenditure elicited by UCP1 activity may have an important role in the body weight control and in the whole-body metabolism. Thus, genetic variations in *UCP1* may affect thermogenesis, and ultimately influence body size and composition, and the risk to develop metabolic disorders. The

A-3826G transition is the most extensively studied *UCP1* polymorphism in human populations. It has been associated with the percentage of body fat gain over time, high weight gain during adult life, and increased BMI in obese subjects (see Jia et al, 2010 for a review). The A-3826G polymorphism is located at the 5' of the gene in proximity of a 350 bp enhancer region (from position -3820 to -3470 upstream of the transcription start site), where T3 (thyroid receptor), fatty acids (PPAR), and retinoic acid (RXR, RAR) response elements are found to be functional (del Mar Gonzales-Barroso et al., 2000). Moreover, a cAMP response element (CRE) is present in this region which is destroyed by a C to A variation at -3737 (Rousset et al., 2002). Interestingly, it has been found that the G -3826 allele is associated with reduced mRNA levels in a dose-dependent manner, indicating its possible functional relevance (Sramkova et al, 2007), Moreover, a strong linkage disequilibrium exists between the A-3826G and the C-3737A polymorphisms (Rousset et al., 2002).

Aging is associated to a decreased ability in the regulation of the energy balance, which contributes to phenomenon of weight and fat losses late in life, an important factor related to the healthy status (Roberts et al., 2006). Indeed, it is well known that body weight chances in older individuals are associated with frailty, functional impairment, and mortality. In this scenario, the A-3826G and C-3737A variants could contribute to the regulation of age-dependent changes in body composition, energy expenditure, and cold thermoregulation, and so may have an effect on the rate and quality of aging. In this study we investigated the influence of the haplotypes/diplotypes constructed from the A-3826G and C-3737A SNPs on human aging and longevity. The functional relevance of the two polymorphisms in the context of haplotypes was also explored.

2. Materials and methods

2.1 Subjects and genotyping

A total of 682 (363 women and 319 men) unrelated individuals (age range: 40-109 years) participated in the present study. The subjects, born and living in Calabria (southern Italy), and whose parents and grandparents were native of the same area, were collected during several campaigns of recruitment (De Rango et al., 2010). All subjects were free of clinically manifested pathologies (cardiovascular diseases diabetes, cancer), and gave their informed consent to use their anonymized genetic and phenotypic data for genetic studies on ageing and longevity.

The UCP1 A-3826G polymorphism was genotyped by genomic PCR and Bcl-I-RFLP analysis following protocols previously described (Oppert et al., 1994). To genotype the UCP1 C-3737A polymorphism 3-5 μl of the same amplified DNA obtained for the other polymorphism was digested by the restriction enzyme DdeI (3 U) for 3 h at 37°C as recommended by the manufacturer. The fragments were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

2.2 Genetic and statistical analysis

$\chi 2$ goodness-of-fit test was applied to the genotype distribution to verify Hardy-Weinberg equilibrium. D' linkage disequilibrium coefficient was assessed using Haploview v3.2 (Barrett et al., 2005). Haplotypes/diplotypes reconstruction was performed with Arlequin version 3.11 for Windows, which implements an EM algorithm and a (Bayesian) ELB algorithm (Excoffier et al., 2005). In order to model haplotype/diplotype frequency as a function of age we carried out a binomial logistic regression analysis, as previously reported by Tan and coworkers (Tan et al., 2001). To this purpose the following model has been proposed:

$$g[\pi_i(x)] = ln\left(\frac{\pi_i(x)}{1-\pi_i(x)}\right) = \beta_{i0} + \beta_{i1}x \qquad (1)$$

In equation (1) $g[\pi_i(x)]$ represents a logit transformation of the frequency of the carrier of the haplotype h_i at age x, $\pi_i(x)$. In such a way, the effect of the haplotype h_i on survival is represented by the slope parameter β_{i1}: if it is significantly different from *0* the frequency of the carrier of the haplotype h_i goes up if $\beta_{i1}>0$ or down if $\beta_{i1}<0$.

As it regard the diplotype frequencies the following model has been proposed:

$$g[\pi_{ij}(x)] = ln\left(\frac{\pi_{ij}(x)}{1-\pi_{ij}(x)}\right) = \beta_{ij0} + \beta_{ij1}x$$

As in the previous case, the effect of the diplotype hi/hj on survival is represented by the slope parameter βij1: if it is significantly different from 0 the frequency of the carrier of the diplotype hi/hj goes up if βij1>0 or down if βij1<0.

the binomial model has been applied to estimate the effect of each of the three reconstructed haplotypes (A-C, G-A and G-C) and the relevant diplotypes on survival.

2.3 Bioinformatic analyses

A 463 bp (corresponding to -3967 to -3504) of the 5′ region of the human *UCP1* gene including both the A-3826G and UCP1 C-3737A polymorphisms was extracted from the National Center for Biotechnology Information data base (http://www.ncbi.nlm.nih.gov/). The search for putative regulatory elements in this sequence was performed using the MatInspector software (http://www.genomatix.de/matins).

2.4 Construction of reporter gene plasmids

The 463-bp region containing the different haplotypes was PCR amplified from human genomic DNA previously sequenced. Primers were designed to include in the product the restriction sites KpnI and SacI for cloning (Forward: 5'caggtaccTAACAGGGTATTTCCCAG; Reverse: 5'cagactCTGAGAGGTCACAGAAGTT). The PCR was carried out in 25 µl containing 1× buffer, 0.2 mM dNTPs, 0.32 µM primers, 2.5 mM MgCl2, and 1.5 U of Go Taq FlexiDNA Polymerase (Promega) (oppure è meglio mettere DNAzyme (Finnzyme)). The PCR reaction consisted of 35 cycles (95°C for 60 s for denaturation step, 55°C for 45 s for annealing step, and 72°C for 60 s for extension step). A denaturation step of 2 min at 95°C preceded the first cycle while the last one was followed by an extension step of 5 min at 72°C. The resulting fragments were purified by agarose gel electrophoresis (Wizard SV Gel; Promega) and digested with KpnI and SacI enzymes (Promega) as recommended by the manufacturer. After the enzymatic digestions, the fragments were purified by phenol extraction and then inserted by using T4 DNA ligase (Promega) into the KpnI-SacI site upstream of the firefly luciferase reporter gene in the pGL3-Basic vector containing an SV40 promoter (Promega). DNA constructs were transformed into Top10 *Escherichia coli* cells by electroporation according to standard protocols. pGL3/UCP1 haplotype constructs were prepared by using the QIAprep Spin Miniprep Kit (Qiagen). The constructs were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) to check the correct insert orientation and to confirm that the sequences matched the original genomic sequences without PCR-generated errors. Cloned vectors were then used for subsequent experiments.

2.5 Transfection assays

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/L glucose and 2 mM L-glutamine supplemented with 5% fetal bovine serum (Invitrogen) and 1% gentamycin (Invitrogen). T47D cells were cultured in RPMI 1640 medium (Sigma) containing supplemented with 4.5 g/L glucose (Sigma), 1mM Sodium pyruvate (Sigma), 10 mM HEPES buffer (Sigma), 0,2 U/ml Insulin (Sigma), 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1% gentamycin (Invitrogen). The cells were cultured in a water-humidified incubator at 37°C in 5% CO2/95% air. Transfections were performed with the Fugene6 reagent as recommended by the manufacturer (Roche Molecular Biochemicals). Briefly, MCF-7 ($5x10^4$) and T47D cells (8×10^4) were transferred into 24-well plates with 1 ml of regular growth medium/well the day before transfection. Then, the growth medium was discarded and replaced with fresh medium supplemented by a mixture containing Fugene6, 1 µg of each reporter plasmid and 1 ng of pRL-CMV (Promega), a plasmid containing the *Renilla* luciferase gene under the cytomegalovirus promoter utilized as an internal control to normalize the effects of transfection efficiencies. After 10 hours, the medium was discarded and incubated in fresh medium containing 17β-Estradiol 1 µM or Progesterone 30 nM or 9-cis Retinoic Acid 1 µM for 48, 36 and 24 hours, respectively.

Then, untreated and treated cells were lysed by applying 100 µl of Passive Lysis Buffer (Promega). 20 µl of cell lysate were used for luciferase reporter assay, by using the Dual Luciferase reporter Assay Kit (Promega), according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activity of the reporter plasmids was normalized to the *Renilla* luciferase activity. Each transfection experiment was carried out three times in duplicate.

Statistical analyses were performed by using SPSS 15.0 statistical software for windows (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) and Student's t-test with a significance level defined as α=0,05, were adopted to check the significance of the difference between the fold induction value of the constructs containing the different haplotypes, at basal conditions and after hormone stimulation.

3. Results

3.1 Haplotype/diplotype analyses

Genotyping of the two SNP sites at the *UCP1* gene was carried out on a sample of 682 subjects categorized according to age and sex-specific classes as reported in Passarino et al (2006). In each group considered there was no evidence for departure from Hardy-Weinberg equilibrium (P>0.05). The two SNPs were in linkage disequilibrium (D'= 0.97), which is not surprising given the close proximity of the two variants. All identified haplotypes and diplotypes are summarized in Table 1 and 2, respectively. A visual inspection of these tables revealed that the distribution of haplotype/diplotype frequencies varied among the age groups. To infer haplotype/diplotype effects on human survival, we modelled the frequency of the carriers of each of the reconstructed haplotype/diplotype as a function of the age by using binomial models.

Table 3 reports the maximum likelihood estimations of the parameters of the fitted haplotype based model in males and females. Of the three reconstructed haplotypes, only the G-A haplotype showed a very significant variation with age in both sexes. In fact, the slope parameter is significantly lower than 0 (β=-0,016) with a corresponding confidence interval ranging from -0,029 to -0,003. If one considers an age interval of fifteen years the corresponding odd ratio (OR) from the fitted model is:

$$OR_{x/x-15} = \frac{e^{\beta_0+\beta_1 x_1+\beta_2 x_2}}{e^{\beta_0+\beta_1(x_1-15)+\beta_2 x_2}} = e^{15\beta_1} = 0{,}852$$

It means that for haplotype G-A carriers a remarkable reduction (around 15%) occurs in an age interval of fifteen years indicating that it is a harmful haplotype. The slope parameters for both A-C and G-C haplotypes are not statistically different from 0, which means that their frequencies are independent of age. Figure 1 shows the frequency patterns for carriers of the G-A haplotype carriers. As expected, both the observed and the fitted frequency patterns indicate there is a constant declining of this haplotype as age goes up.

In order to check if the detrimental effect of the G-A haplotype observed in both sexes did involve a particular diplotype, we analyzed the frequency of the carriers of each of the six reconstructed diplotypes as a function of age by using the model (2).

Table 4 reports the parameter estimates for all the reconstructed diplotypes. The diplotype A-C/A-C showed a highly significant slope parameter (β=0.012; p=0.005). The positive slope parameter indicates that the frequency of subjects with this diplotype increases with age. The diplotype A-C/G-A showed also an highly significant slope parameter (β=-0.016; p=0.000). The negative slope parameter indicates that the frequency of subjects with this diplotype decreases with age (Figure 2). As for the haplotypes, if one considers an age interval of the years the corresponding ORs from the corresponding fitted models are 1.127 and 0.852 for the diplotype A-C/A-C and A-C/G-A respectively. The diplotype A-C/G-C did not show any significant variation with age. The others diplotypes failed to reach the level of significance probably due to the low frequencies. On the whole, these results indicate that the haplotype A-C has a recessive and beneficial effect, while the haplotype G-A has a dominant and deleterious effect.

3.2 Functional analyses of haplotypes

To ascertain whether the observed associations reflected a different functional relevance of the haplotypes, we first submitted a sequence of 463bp (from -3967 to -3504) which included the two polymorphic sites to the MatInspector online software. As shown in Figure 3, a putative retinoic acid response element (from -3842 to -3826), including the A-3826G variation, and a previously characterized ATF/CREB binding element (from --3738 to -3733), which is modified by C-3737A variation (Rousset et al., 2002), were predicted. Furthermore, a progesterone responsive element (PRE)-like sequence (from.-3817 to -3804), and an estrogen responsive element (ERE)-like sequence (from -3713 to -3701), were also predicted in the vicinity of the two SNPs. Subsequently, we generated reporter constructs carrying the three haplotypes and tested their transcriptional activity in transiently transfected MCF-7 and T47D cells, which specifically express the estradiol and progesterone receptors respectively, using a luciferase reporter gene assay. Experiments were carried out both under basal condition and after hormonal stimulation. As can be seen from Figure 4, in both cell lines under basal condition, the luciferase activity of the cells carrying either the A-C or the G-A construct were significantly higher than those of the cells carrying the haplotype G-C ($p= 0.005$ and $p= 0.007$ respectively), which actually did not show any activity. Moreover, the activity of the A-C construct was higher than that of G-A ($p= 0.017$). A major effect of one of the two polymorphisms was not apparent following analysis of all combination of haplotypes.

With respect to basal condition, a higher luciferase activity was observed for both the A-C ($p=0.027$) and G-A ($p= 0.046$) constructs in MCF-7 cells treated with 9-cis retinod acid (1µM) for 24h in (Figure 4a). The activity of the sole A-C construct increased ($p=0.0149$) instead when T47D cells were treated with progesterone (30nM) for 36h

(Fgure 4b). In contrast, A-C demonstrated a significantly decreased luciferase activity ($p=0.028$) when MCF-7 cells were treated with 17β-estradiol (1 μM) for 48h (Figure 4c). In presence of 17β-estradiol also the G-A construct was down-regulated but not a statistically significant level compared to basal transcription.

4 Discussion

The present study provides evidences that the variability of the BAT-specific *UCP1* gene is associated with human aging and longevity. Early studies in rodents have demonstrated that BAT and UCP1 activity have a role in energy balance and in body weight control. BAT-deficient mice are obese and exhibit reduced energy expenditure and insulin resistance (Hamann et al., 1998); likewise, ablation of *UCP1* induce attenuation of fatty acid utilization and non-shivering thermogenesis, and obesity (Kontani et al., 2005; Feldmann et al., 2009). Moreover, in rodents the decline of thermogenesis during aging contributes to weight gain and visceral adiposity (McDonald and Horwitz, 1999), two phenomena with a significant pathophysiological role in the development of the metabolic syndrome. Consistently, mice ectopically expressing *UCP1* showed an accelerated metabolism and also displayed increased median survival and diminished age-related disease (Gates et al., 2007).

Human aging is accompanied by alterations in body composition, including reduction in lean body mass (Gallagher et al., 2000) and increase in adiposity (Hugheset al., 2004)., consequent to hormonal changes associated with menopause in women and adrenopause in men The possibility that the thermogenic activity of BAT can protect human body against the aging process is suggested by some evidences. For instance, BAT-mediated responsiveness to cold temperature declines with age, and an inverse relation between BAT activity and BMI, body fat, and visceral fat has been observed (Saito et al., 2009; Mattson, 2010). These findings point to a relevant role of BAT in the control of

adiposity via the regulation of energy expenditure in humans. In fact, although the amount of BAT in human adults is low, it is however responsible for 1-2% of the energy expenditure, which is enough to prevent a weight gain of about 2 kg per year (Lean, 1989). A number of nucleotide variants in the *UCP1* gene have been described and analyzed in many conditions associated with human aging. In addition to the A-3826G variation, the A-1766G and A-112C at the 5'-flanking region and the Ala64Thr in exon 2 polymorphisms are associated with body fat accumulation and body weight gain or body mass index (BMI) in Caucasian and East Asian populations (Hamann et al., 1998; Herrmann et al., 2003; Kim et al., 2005; Kim et al., 2006). Moreover, two polymorphisms in exon 1 and 5 were both associated with susceptibility to type -2 diabetes (Mori et al., 2001), all of which are indicative for a role for the variations in the *UCP1* gene in body weight regulation in humans Our finding of a constant age related increase of the A-C/A-C diplotype carriers' frequency and of a consequent decline of the A-C/G-A diplotype carriers' frequency, provide additional indications on a role of UCP1 in aging. In fact, it suggests that that the variability of *UCP1* may modulate the different metabolic processes, such as fat metabolism and energy expenditure, which are crucial for determining survival at very old age.

As to the mechanisms explaining this effect, the results of our in vitro transfection experiments using luciferase reporter assay clearly point to an effect of these haplotypes on *UCP1* gene expression. The A-C haplotype, which is beneficial for human survival, showed a higher transcriptional activity compared to the detrimental G-A haplotype. This suggests a correlation between *UCP1* gene expression and survival. It is questioning why the G-C haplotype, that did not show any significant changes in its frequency during aging, also did not show any transcriptional activity. This finding suggests a complex mechanism of transcriptional regulation of the *UCP1* gene that may

depend on the activity of the entire region. As previously mentioned in the introduction, the A-3826G and the C-3737A polymorphisms we analyzed are located in an enhancer region of the gene containing multiple and distinct cis-acting elements that mediate a strong drug-dependent transcriptional activation of the *UCP1* gene (del Mar Gonzalez-Barroso et al, 2000). Our bioinformatic analysis showed that the A-3826G occur in a putative retinoic acid-response element, and that a putative Progesterone-Response Element (PRE) and a putative Estrogen-Response Element (ERE) are also present in the vicinity of the two polymorphisms. Intriguingly, we found that different *UCP1* haplotypes differently respond to hormonal stimuli displaying a complex pattern of functional effects. The up-regulation following retinoic acid treatment is consistent with the presence of several putative RARE elements in the cloned sequence and confirms retinoic acid as one of the major regulators of the *UCP1* gene transcription (Alvarez et al., 2000). No substantial differences were observed in the retinoic acid transcriptional activation between the A-C and G-A haplotypes.

The presence of consensus sex steroid responsive elements is very intriguing. Indeed, there are several reports considering sex steroid hormones as modulator of the thermogenic capacity and activity of BAT (Monjo et al., 2003; Valle et al., 2008). We showed that the A-C haplotype was significantly up-regulated in presence of progesterone and down-regulated in presence of estradiol. This result leads to two considerations. First, the different effect of the *UCP1* haplotypes could be explained by taking into account the age-related changes in fat storage and fat metabolism, possibly mediated by the age-related variations in the hormonal status. Second, as suggested by Fessele et al. (2002), the importance of the sequence context in determining the functionality of a regulatory region clearly emerge. In fact, although the two polymorphisms did not alter neither the binding site for progesterone nor the binding

site for estradiol, a clear haplotype-specific effect on the transcriptional activity is observed following hormonal stimulation.

5. Conclusions

Our study provides the evidences for haplotypic variants in the 5'-enhancer of *UCP1* gene in modulating survival, probably by affecting the levels of the protein. This is the first study reporting a significant association between *UCP1* genetic variants and human survival and additional data are needed. On the other hand the soundness of our findings is supported by the consistency between our association and in vitro studies and previous studies in model organisms.

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Figure 1. The observed and the estimated frequency for haplotype G-A carriers.

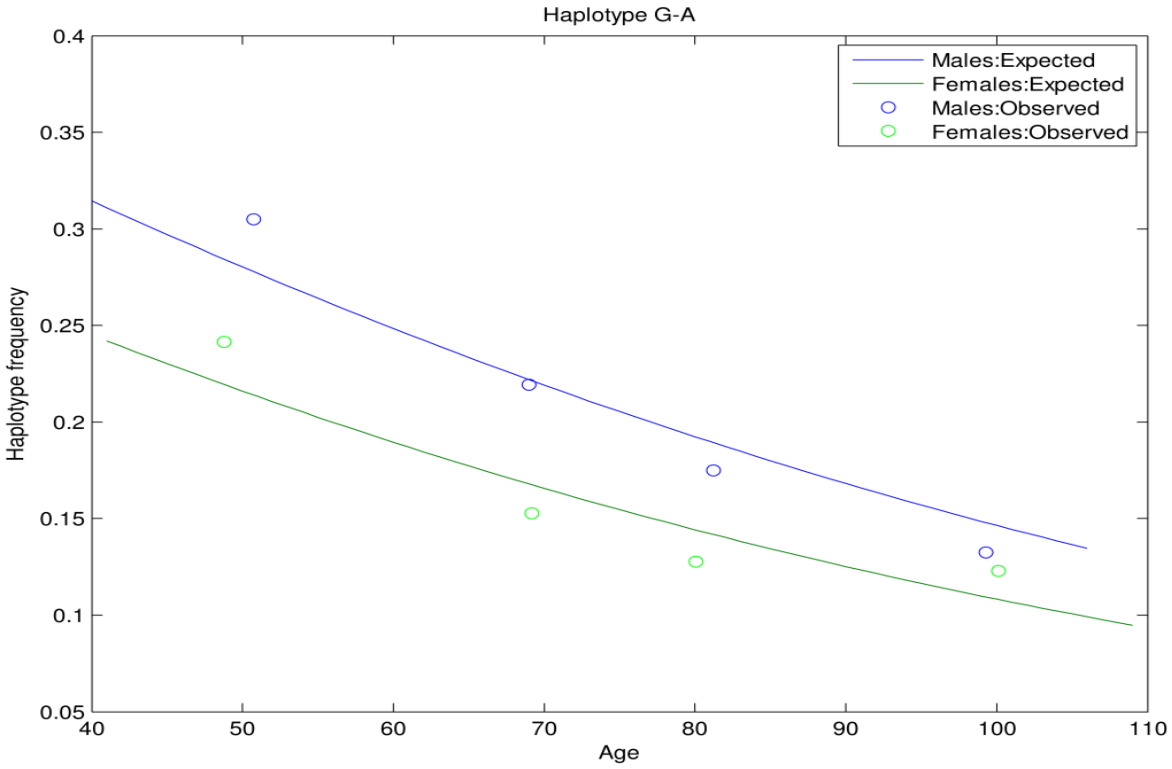
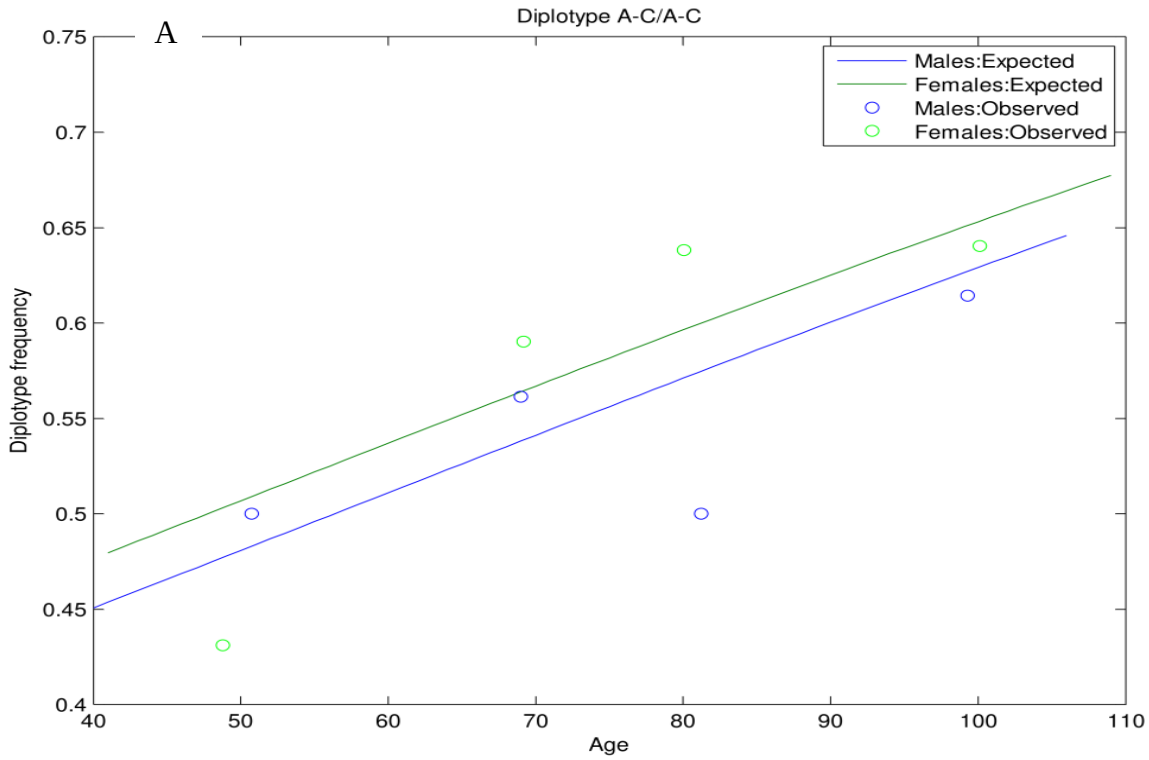
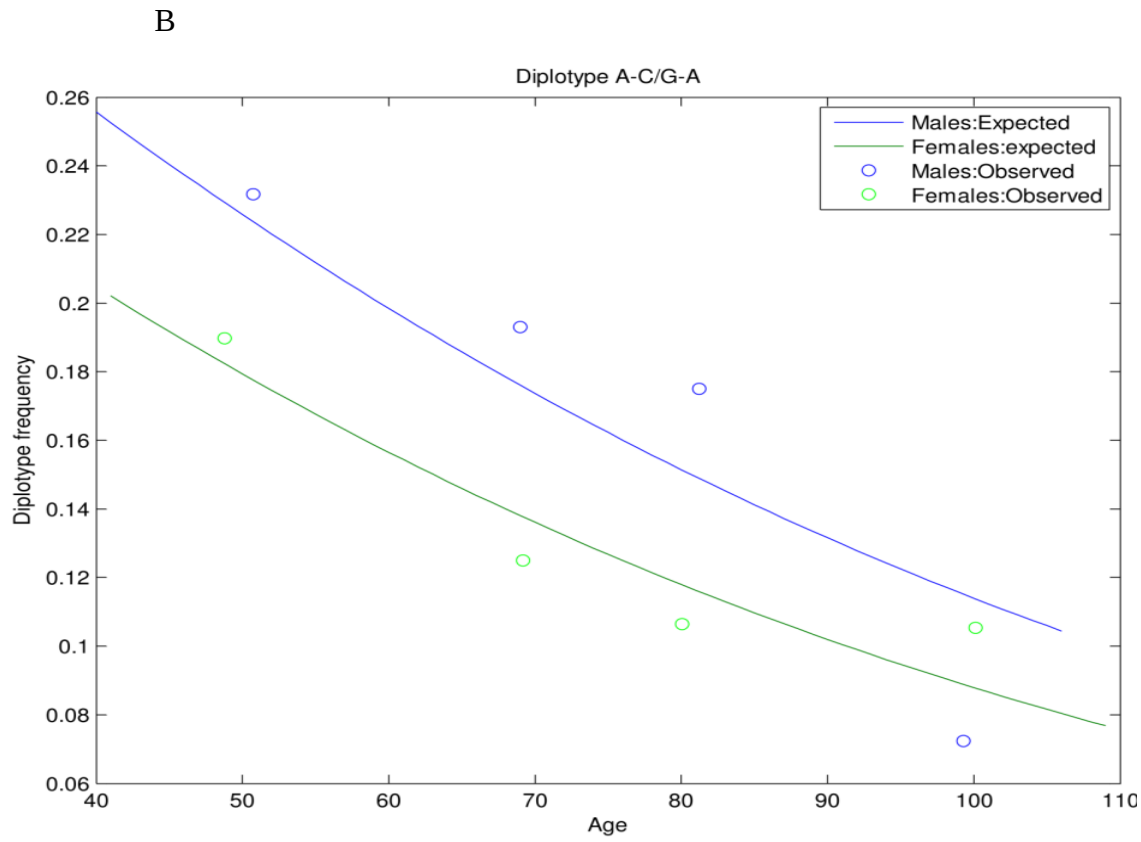


Figure 2. The observed and the estimated frequency for diplotype A-C/A-C (A) and A-C/G-A (B) carriers.





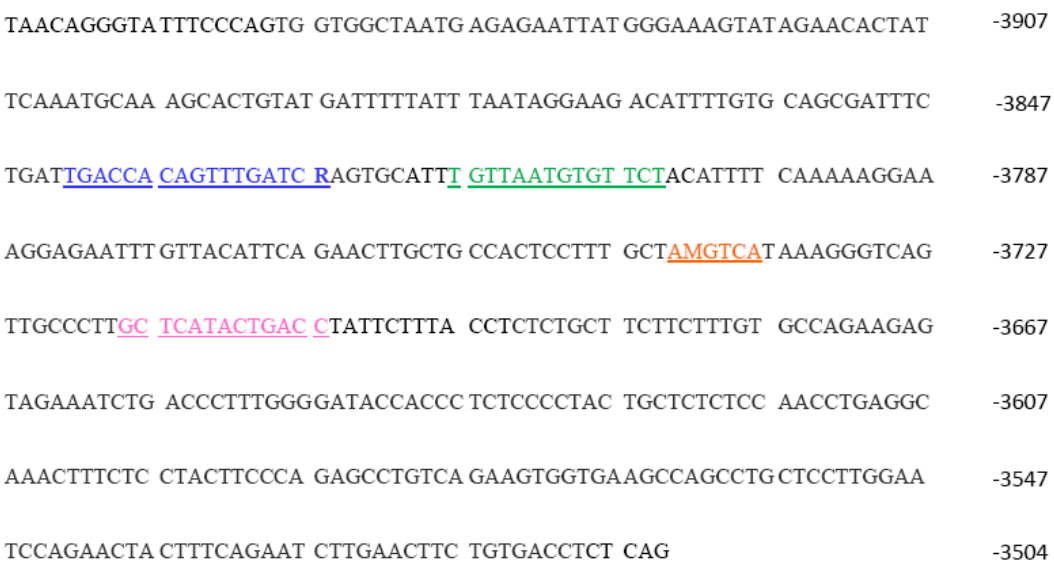
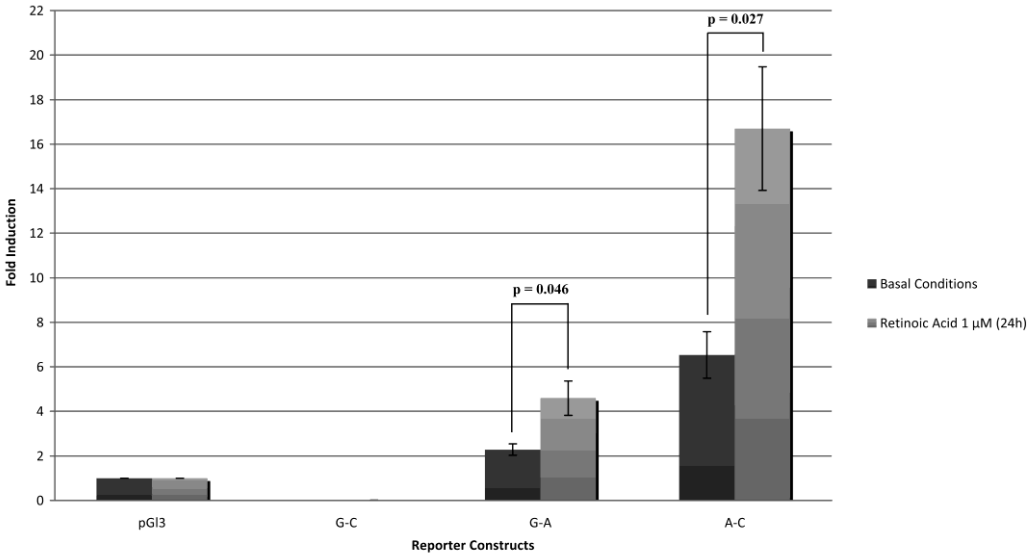
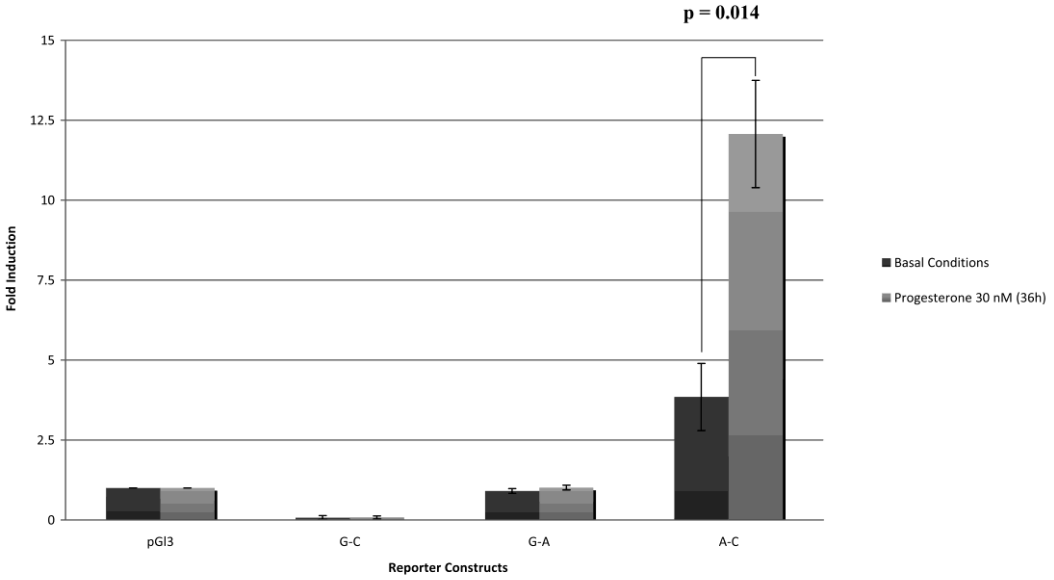


Figure 3. Cloned sequence of the UCP1 gene containing the A-3828G (R) and the C-3737A (M) polymorphisms. Retinoic Acid-Response Element (blue), Progesterone-Response Element (green), ATF/CREB binding element (orange), and Estrogen-Response Element (pink) are underlined.

A


p = 0.027
p = 0.046
Fold Induction
22
20
18
16
14
12
10
8
6
4
2
0
pGl3
G-C
G-A
A-C
Reporter Constructs
Basal Conditions
Retinoic Acid 1 µM (24h)

B


p = 0.014
Fold Induction
15
12.5
10
7.5
5
2.5
0
pGl3
G-C
G-A
A-C
Reporter Constructs
Basal Conditions
Progesterone 30 nM (36h)

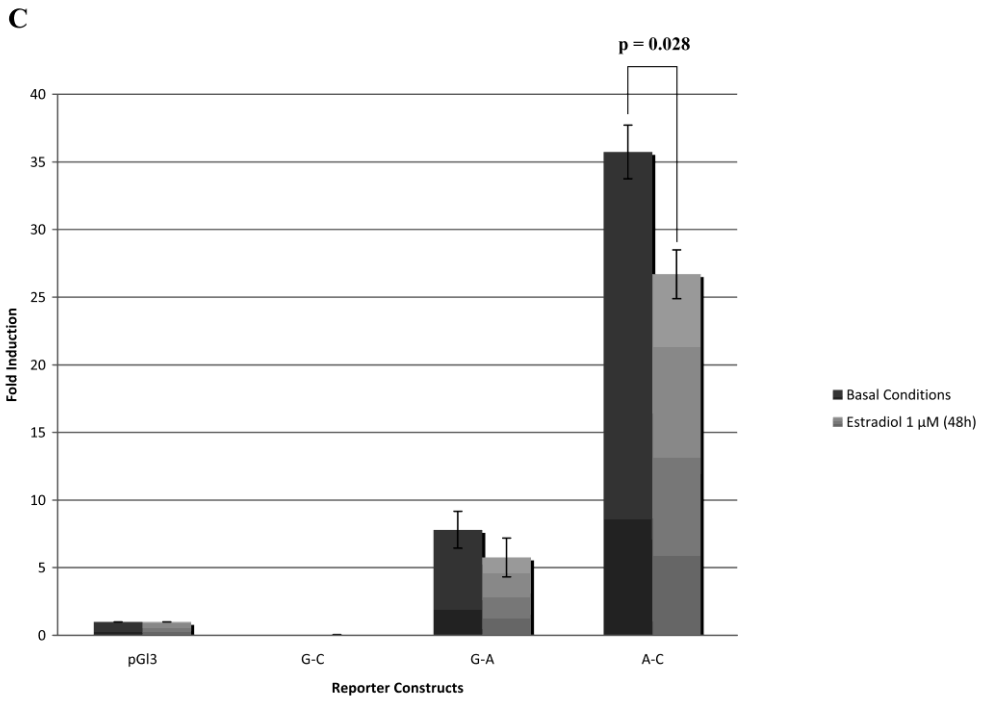


Figure 4: Luciferase activity of the reporter constructs at basal and after stimulation.A) MCF-7 cells treated with 1 µM of Retinoic Acid for 24 hours; B) T47D cellstreated with 30 nM of Progesterone for 30 hours; C) MCF-7 cells treated with 1 µM Estradiol for 48 hours. The values reported are the means ± standard error mean of three independent experiments.

Table 1. Frequency distribution of two-marker haplotypes in the UCP1 region in the analyzed sample categorized according to the defined age classes. Standard errors (SE) of haplotype frequencies were calculated through 1000 bootstrap resampling cycles.

	Age groups		
	Females		
Haplotypes	**Age Class 1**	**Age Class 2**	**Age Class 3**
	2n=334	2n=166	2n=226
	(40-72 years)	(73-91 years)	(>91 years)
	Rel. Freq. ± SE	Rel. Freq. ± SE	Rel. Freq. ± SE
A-C	0.728±0.025	0.801±0.032	0.801±0.027
G-A	0.099±0.016	0.072±0.021	0.062±0.016
G-C	0.174±0.021	0.127±0.026	0.137±0.023
A-A	-	-	-
	Males		
Haplotypes	**Age Class 1**	**Age Class 2**	**Age Class 3**
	2n=208	2n=256	2n=174
	(40-65 years)	(66-88 years)	(>88 years)
	Rel. Freq. ± SE	Rel. Freq. ± SE	Rel. Freq. ± SE
A-C	0.716±0.031	0.742±0.028	0.776±0.032
G-A	0.144±0.024	0.113±0.020	0.075±0.020
G-C	0.139±0.024	0.145±0.021	0.149±0.026
A-A	-	-	-

*Testing for independence has been performed by a Monte Carlo approximation to Fisher's exact test (Agresti et al., 1979).

*Based on 10000 sampled tables

Table 2. Diplotype frequency distribution in the analyzed sample categorized according to the defined age classes..

	Females			Males		
Diplotype	**Age Class 1** N=167 (40-72 years)	**Age Class 2** N=83 (73-91 years)	**Age Class 3** N=113 (>91 years)	**Age Class 1** N=104 (40-65 years)	**Age Class 2** N=128 (66-88 years)	**Age Class 3** N=87 (>88 years)
A-C/A-C	86 (51.5%)	54 (65.1%)	73 (64.6%)	54 (51.9%)	68 (53.1%)	54 (62.1%)
A-C/G-A	26 (15.6%)	9 (10.8%)	11 (9.7%)	20 (19.2%)	27 (21.1%)	7 (8.0%)
A-C/G-C	45 (26.9%)	16 (19.3%)	24 (21.2%)	21 (20.2%)	27 (21.1%)	20 (23.0%)
G-A/G-A	2 (1.2%)	0 (0.0%)	1 (0.9%)	3 (2.9%)	0 (0.0%)	1 (1.1%)
G-A/G-C	3 (1.8%)	3 (3.6%)	1 (0.9%)	4 (3.8%)	2 (1.6%)	4 (4.6%)
G-C/G-C	5 (3.0%)	1 (1.2%)	3 (2.7%)	2 (1.9%)	4 (3.1%)	1 (1.1%)

*Testing for independence has been performed by a Monte Carlo approximation to Fisher's exact test (Agresti et al., 1979).

*Based on 10000 sampled tables

Table 3. Maximum likelihood estimations of the parameters of the fitted binomial logistic regression models in the analyzed sample.

Haplotype A-C				
Papameter	**β**	**SE**	**P-value**	**95% CI**
Age	0.009	0.009	0.343	-0.009 - 0.026
[Sex=Males]	-0.216	0.328	0.511	-0.858 - 0.427
Constant	2.252	0.708	0.001	-
Haplotype G-A				
Age	-0.016	0.006	0.003	-0.027 - -0.005
[Sex=Males]	0.346	0.201	0.086	-0.049 - 0.741
Constant	-0.470	0.434	0.279	-
Haplotype G-C				
Age	-0.004	0.005	0.439	-0.013 - 0.006
[Sex=Males]	-0.072	0.173	0.679	-0.411 - 0.268
Constant	-0.674	0.378	0.074	-

Table 4. Maximum likelihood estimations of the parameters of the fitted binomial logistic regression models in the analyzed sample.

Diplotype A-C/A-C				
Papameter	**β**	**SE**	**P-value**	**95% CI**
Age	0.012	0.004	0.005	0.004 - 0.020
[Sex=Males]	-0.104	0.157	0.506	-0.411 - 0.203
Constant	-0.578	0.343	0.092	-
Diplotype A-C/G-A				
Age	-0.016	0.006	0.007	-0.028 - -0.004
[Sex=Males]	0.289	0.219	0.188	-0.141 – 0.718
Constant	-0.702	0.471	0.136	-
Diplotype A-C/G-C				
Age	-0.003	0.005	0.588	-0.013 – 0.007
[Sex=Males]	-0.130	0.185	0.483	-0.493 – 0.233
Constant	-0.977	0.403	0.015	-
Diplotype G-A/G-A				
Age	-0.022	0.022	0.307	-0.065 – 0.020
[Sex=Males]	0.349	0.771	0.651	-1.163 – 1.860
Constant	-3.152	1.630	0.053	-
Diplotype G-A/G-C				
Age	-0.007	0.013	0.625	-0.033 – 0.020
[Sex=Males]	0.476	0.501	0.342	-0.505 – 1.458
Constant	-3.429	1.080	0.001	-
Diplotype G-C/G-C				
Age	-0.004	0.014	0.778	-0.031 – 0.023
[Sex=Males]	-0.138	0.512	0.787	-1.142 – 0.865
Constant	-3.374	1.101	0.002	-

3. A common UCP3 promoter polymorphism influences hand grip strength in elderly people (Submitted to Biogerontology)

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Abstract

The reduction of muscle mass in the elderly is widely studied as one of the most important landmark of human aging. This process, which occurs for the decay of the correct energetic and protein metabolism, has important functional consequences on individual functionality but also on metabolic adaptation and immunological response to environmental challenges.

Uncoupling Protein 3 (*UCP3*) gene is expressed in skeletal muscle where it regulates fatty acid metabolism, redox state, and ROS formation. Considering the importance of these process in aging, we studied two variants of the *UCP3* gene in a large aged (age range 65-105) population of southern Italy verifying if these variants were correlated to hand grip strength, the most reliable measure of muscle decay.

We found that a previously described functional polymorphism, rs1800849, located in the promoter region of the *UCP3* gene, has a significant impact on hand grip strength. In fact, the carriers of rs1800849 T allele showed higher hand grip than the remaining of the population. Since this allele has been reported to promote a higher expression of the gene, we conclude that a more efficient uncoupling process has a beneficial effect on the aging muscle by slowing down its age related decay.

Key words: Uncoupling protein; hand grip strength; aging.

Introduction

During the aging process a significant loss of muscle mass and function occurs in skeletal muscle of a variety of mammalian species (Lee et al. 1993; Cortopassi and Arnheim 1990; Linnane et al. 1990; Melov et al. 1994; Chung et al. 1994). This process is referred to as sarcopenia. In humans a significant and gradual loss of skeletal muscle after the fifth decade of life has been observed (Carmeli et al. 2000; Doherty 2003). However, if on one hand it is widely well known that the reduction in lean body mass has important functional consequences, on the other hand a number of physiological functions that take place within muscle tissues are equally important. Indeed, muscles are an important reserve of body proteins and energy that can be used in extreme conditions of stress or malnutrition; amino-acids can be mobilized during acute infections and used as building blocks for antibodies; hormones are produced and catabolized within muscle tissue. Thus, it is not surprising that the reduction in muscle mass has a negative impact on metabolic adaptation and immunological response to disease, therefore reducing the overall homeostasis of the elderly and his ability to resist environmental challenges (Schrager et al., 2003).

Although the concept of sarcopenia is frequently used in research settings and introduced to clinical settings, no consensus on its definition has been established (Visser 2009). For this and other practical reasons the age-related loss of skeletal muscle mass is usually operationalized as low muscle strength in old age. It is usually measured as hand grip strength that resulted to be strongly correlated with other measures of muscle strength and therefore is often considered representative of total body muscle strength. Different studies showed that hand grip strength is predictive of disability (Rantanen et al. 1999; Giampaoli et al. 1999) and mortality (Metter et al.

2002; Rantanen et al. 2003; Newman et al. 2006). Indeed, sarcopenia is considered one of the major contributor to frailty (Morley et al. 2001).

It is then evident that elucidating the pathophysiological mechanisms underlying sarcopenia remain not clear, their understanding might have important socio-economic (Janssen et al. 2004) and public health (Roubenoff 2000a) implications. Indeed, the functional limitations and impairments due to sarcopenia reduce quality of life and compromise functional independence throughout senescence (Janssen et al. 2002). Moreover, sarcopenia and other related conditions such as malnutrition may be amenable to health interventions (Roubenoff 2000b).

To date, there is a growing consensus that sarcopenia is a multifactorial condition caused by atrophy and loss of muscle fibers (Lexell et al. 1988; Lexell 1995) for which several mechanisms have been proposed. These include inadequate dietary protein and caloric intake (Dardevet et al. 2000), decreasing physical activity (Nelson et al. 1994; Fiatarone et al. 1994; Jozsi et al. 1999), deficient satellite cell recruitment (Carlson 1995), denervation/renervation (Larsson 1995; Lexell 1997), endocrine changes (Balagopal et al. 1997), development of several catabolic stimuli (Roubenoff and Hughes 2000; Roubenoff et al. 2003; Doherty 2003; Roubenoff 2003), such as inflammatory cytokines, interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), oxidative stress (Cesari et al. 2004; Meng and Yu, 2010) and mitochondrial DNA (mtDNA) damage (Cortopassi et al. 1992; Wanagat et al. 2001; Meissner et al. 2006). It is generally accepted that the latter two mechanisms (oxidative stress and mitochondrial damage) contribute, more or less in concert, to this progressive age-related loss of muscle mass (McKenzie et al. 2002).

To date five mitochondrial uncoupling proteins (UCPs) have been identified in human. These proteins seem to play an important role in the regulation of Reactive Oxygen

Species (ROS) formation. In fact, due to their localization in the mitochondrial inner membrane, they cause the proton gradient dissipation, uncoupling respiration from ATP synthesis (Yu et al. 2000). Among five UCPs, the mitochondrial uncoupling proteins 3 (UCP3) is mainly expressed in skeletal muscle where it regulates fatty acid metabolism, redox state, and ROS formation (Boss et al. 1997; Echtay et al. 2002). Moreover, UCP3 could play an important role in energy metabolism. Indeed, several studies showed that dietary alterations (Weigle et al. 1998), thyroid hormones (Larkin et al. 1997) and agonists of the b-3 adrenergic receptor (Gong et al. 1997) are among UCP3 regulatory elements. Due to these mechanisms, UCP3 may be considered such as a candidate gene for sarcopenia phenotype.

Among the several polymorphisms of *UCP3* gene, which have been described, we analyzed two variants: a functional promoter region variant rs1800849 (Schrauwen et al. 1999) and a linked 3'UTR variant (rs15763) with a still unclear functional role. The aim of this study was to determine whether genetic variations in the *UCP3* gene influence hand grip strength in elderly subjects .

Materials and Methods

Sample

The sample analyzed included 65–105 years old subjects (432 subjects, 221 males and 211 females; median ages 79.5 and 82.0 years, respectively). All the subjects were born in Calabria (southern Italy) and their ancestry in the region had been ascertained up to the grandparents' generation. The sample has been recruited in the frame of different recruitment campaigns carried out by our research group between 2002 and 2007. Subjects older than 90 years were identified through the population registers and then

contacted by specialised personnel and invited to join the study. Younger subjects were contacted either through general physicians or by means of the I. N. R. C. A. Hospital, which is a reference point for the care of the aging people in the Calabria region. Finally, each subject was met by a geriatrician and a person (usually a biologist) trained to conduct a structured interview. All the subjects were recruited after a complete multidimensional geriatric assessment with detailed clinical history, including anthropometric measures and a set of the most common tests to assess cognitive functioning, functional activity, physical performance and depression. In addition, common clinical haematological tests were performed. Subjects with dementia and/or neurologic disorders were not included. Phenotypic information was collected by using the questionnaires available at the following web site: http://biologia.unical.it/echa/results.htm. All the subjects had given informed consent for studies on aging carried out by our research group.

Measurement of hand grip strength and anthropometric characteristics .

Hand grip strength was measured by 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; the maximum of these values was used in the analyses. When a test was not carried out, it was specified if it was due to physical disabilities or because the subject refused to participate.

Height and weight were measured in the frame of the multidimensional geriatric assessment, and Body Mass Index (BMI) was calculated as weight (in kilograms) divided by the square of height (in meters).

Genotyping

Genotyping of the two polymorphic sites was carried out using a TaqMan Real Time PCR (SNP Genotyping kit, Applied Biosystems). In both assays, the fluorescent FAM dye was used to label the wild-type allele, while the fluorescent VIC dye was used to label the other one. Genotyping was performed by analyzing the fluorescent pattern of each sample.

PCR reactions were carried out in a total volume of 5 μl contained 20 ng of genomic DNA, 2.5 μl of TaqMan Universal Master mix (concentration of 2x), 0.25 μl of Custom TaqMan SNP Genotyping Assay (concentration of 20x) containing both primers and probes.

The amplification protocol (60 °C for 30 seconds and then 95 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute) was performed by using a StepOne thermal cycler (Applied Biosystems). Random regenotyping were conducted to confirm the results.

Statistical analysis

UCP3 allele frequencies were estimated by counting genes from the observed genotypes. The Hardy-Weinberg equilibrium (HWE) was tested by using a Monte-Carlo approach based on 10,000 random allele permutations for each sample (Weir 1996). Standard errors for alleles was computed according to the hypothesis of the multinomial distribution.

An expectation maximization (EM) algorithm, implemented in the Arlequin software package (Excoffier et al. 2005) was used to obtain maximum likelihood estimation of multilocus haplotype frequencies for UCP3 genotypes. Standard deviations of the

haplotype frequencies were estimated by a parametric bootstrap procedure (1000 replications). Pairwise linkage disequilibrium was tested by using a likelihood ratio test in which the likelihood of the observed data evaluated under the hypothesis of no association between loci is compared to that evaluated under the hypothesis of association. Haplotypes were constructed based on the population genotypes in the UCP3 gene using a pseudo-Bayesian approach (ELB algorithm) implemented in the Arlequin software package (Excoffier et al. 2005).

Multiple regression was used to investigate the relationship between hand grip strength measures and UCP3 genotypes/haplotypes. In particular, hand grip strength was used as dependent variable, age, sex and height were used as covariates and UCP3 genotypes/haplotypes as a factor. The interactions between covariates and UCP3 genotypes/haplotypes were included in the model in order to test whether the slopes of the regression lines were the same in the analyzed subgroups . Each SNP was analyzed independently of each other. A significance level of 0.05 was chosen in all the tests. Statistical analyses were performed by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 reports the socio-demographic characteristics of the analyzed sample, together with the information on hand grip measurements. The frequencies of *UCP3* genotypes and alleles in the sample are reported in Table 2. No significant deviation from HWE was detected for both the two *UCP3* SNPs analyzed. The genotype frequencies for variant allele homozygotes were low, so they were combined with the heterozygotes into one group as variant allele carriers for both the two SNPs in the following data analyses. The pairwise linkage disequilibrium between the two selected polymorphisms

of *UCP3* gene was assessed and a strong allelic association was detected (D'=0.82 p<0.001).

In order to explore the influence of the *UCP3* polymorphisms on the physical performance, we analyzed the association between *UCP3* genotypes/haplotypes and hand grip scores. No significant differences for hand grip values were found between genotype groups of the rs15763 variant (CC *vs* CT+TT, p=0.210). on the contrary, significant differences for hand grip values were found between genotype groups of rs1800849 variant (CC *vs* CT+TT, p=0.010). In particular, carriers of the rare allele of this polymorphism showed higher hand grip than the relevant wild-type homozygote genotype carriers (Table 3).

In a second step we evaluated whether the reconstructed *UCP3* haplotypes of the selected polymorphisms exert effects on the hand grip performances. We found that the haplotypes containing the allele showing a significant single-locus association also showed higher hand grip performances than the remaining haplotypes (data not shown).

Discussion

Hand grip strength is widely recognized as one of the most reliable markers of the physiological status of the elderly, as it reflects the status of the muscle mass, which in turns reflects a complex equilibrium between nutrition, metabolism and the response to external stress. In fact, it has been recognized as the most effective death predictor in the elderly (Metter et al. 2002; Rantanen et al. 2003; Newman et al. 2006). The survey of hand hrip strength in large population samples, including the children of the centenarians has shown that hand grip is partly determined by individual genetic background. In particular, the analysis of monozygotic and dizygotic twins has shown that about 50% of the observed variation in hand grip strength could be explained by additive genetic effects (Frederiksen et al. 2002). Thus, it has been suggested that hand grip strength maybe suitable for identifying genetic variants affecting mid- and late-life physical functioning. Indeed, a number of genetic variants in relation to this phenotype have been identified (Arking et al. 2006; Roth et al. 2001; Dato et al. 2010; Ronkainen et al. 2008; Walsh et al. 2009; Walsh et al. 2008; De Mars et al. 2007). In the present study we show that the variability of *UCP3* rs1800849, located in the promoter region of the gene, affects hand grip strength in an elderly population from southern Italy. In particular, the carriers of T allele (TT and CT) show significantly higher hand grip strength with respect to the carriers of the CC genotype. This suggests a correlation between the uncoupling process and the regulation of muscle metabolism/catabolism that takes place in the elderly. In particular, as previous reports showed that the T allele increases UCP3 mRNA expression in skeletal muscle compared with the C allele (Schrauwen et al. 1999), our findings indicate that an increased UCP3 activity in skeletal muscle might slow down the age related decline of muscle mass and performance. Many reports have suggested the possibility that an efficient uncoupling

process may have a beneficial effect on the age related decay of the senescent tissues (Nabben et al. 2008; Mookerjee et al. 2010). This may be due to a more efficient energy metabolism related to the uncoupling process that in turn reduces production of ROS which have a protective effect in lipid peroxidation and of mitochondria. Our findings are fully in keeping with this hypothesis.

It may be worthy noting that the impact of *UCP3* variability on the aging muscle we report may suggest a reappraisal of the effect of UCPs on the aging tissues. In fact, if *UCP3* is expressed in the muscle tissue, additional UCPs are expressed in different tissues (*UCP1* in brown adipose tissue; *UCP2* is widely expressed in many organs and tissues such as pancreas, white adipose tissue, lung, spleen, brain; *UCP4* and *UCP5* are expressed predominantly in the central nervous system and at a lower level in other tissues). It is then possible that also the variability of different UCPs may affect the senescence of different tissues with important effects on the overall aging process and then to have an important role in human longevity.

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Table 1. Socio-demographic characteristics and hand grip strength measurements of the analyzed sample by sex.

Variables	**Men**	**Women**	**P-value**
Age	79.99 (12.14)	81.82 (12.17)	0,08125
Weight (Kg)	70.97 (14.79)	60.50 (14.18)	<0.001
Height (m)	1.65 (0.08)	1.52 (0.08)	<0.001
BMI (Kg/m^2)	26.03 (4.28)	25.97 (5.09)	0,625
Hand Grip strength (Kg)	25.21 (10.23)	14.64 (6.28)	<0.001

Table 2. Absolute (N) and relative (%) genotypic and allelic frequencies of the UCP3 polymorphisms in the analyzed sample.

UCP3 Polymorphism rs15763

		N	%	SE	P-value (HWE)
Genotypes	C/C	249	57.6%	2.4%	
	C/T	157	36.3%	2.3%	
	T/T	26	6.0%	1.1%	0,6625
		N	%	SE	
Alleles	C	655	75.8%	1.5%	
	T	209	24.2%	1.5%	

UCP3 Polymorphism rs1800849

		N	%	SE	P-value (HWE)
Genotypes	C/C	363	84.0%	1.8%	
	C/T	66	15.3%	1.7%	
	T/T	3	0.7%	0.4%	0.999*
		N	%	SE	
Alleles	C	792	91.7%	0.9%	
	T	72	8.3%	0.9%	

*Pearson's Chi-squared test with simulated p-value (based on 10000 replicates)

Table 3. Adjusted means for hand grip strength by UCP3 genotypes in the analyzed sample.

UCP Polymorphisms	**Hand Grip strength**	**P-value**
UCP3 – rs15763 SNP1		
Carriers CT+TT	20.541 (0.478)	
Carriers CC	19.738 (0.404)	0,1395833
UCP3 – rs1800849 SNP2		
Carriers CT+TT	21.912 (0.773)	
Carriers CC	19.728 (0.334)	0.010

The reported values are least square means (standard errors in parentheses)
Hand grip strength measures were adjusted for age, sex and height.

4. Further support to the Uncoupling-to-Survive theory: the genetic variation of human UCP genes is associated with longevity (Submitted to Mechanism of ageing and Development)

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Abstract

The uncoupling of respiration from ATP production, is a mitochondrial process by which stored energy is released as heat. In human, uncoupling is mediated by a group of five mitochondrial inner membrane transporters, known as uncoupling proteins (UCP), which seem to function as regulators of energy homeostasis and as antioxidants. Thus, a role of UCPs in human ageing and longevity has been suggested.

In order to study the involvement of UCP genes in human ageing, we screened 621 unrelated southern Italian individuals (age range: 64-105 years) for ten SNPs of UCP1-5 genes. A total of, were. We set up a multivariate logistic-regression model to evaluate the effects of UCP genotypes on the probability to reach advanced ages. We found that UCP1, UCP3, and UCP4 genetic variation affects male chance to survive to very old ages, while in females only the UCP3 affects such chance. Our results suggest that the UCPs gene variability modulates survival at old ages in a gender-specific way. As UCPs are differently expressed in various organs and tissues, our results allowed some inferences on the involvement of the uncoupling process (and of energy storage and expenditure) in the elderly.

Key words: Uncoupling proteins; mitochondria; energy storage; longevity.

Introduction

Un-Coupling Proteins (UCPs) belong to a family of anion transporters located in the inner membrane of mitochondria, that uncouple substrate oxidation from ATP synthesis. As a consequence stored energy is released as heat. To date, five UCP homologues, *UCP1* to *UCP5*, have been identified in mammals (Yu et al., 2000). UCP1 (4q28.31), the first member characterized, is predominantly found in brown adipose tissue (BAT), where it has a well established role in cold- and diet-induced thermogenesis (Cannon and Nedergaard, 2004). UCP2 and UCP3 are closely located on chromosome 11q13. UCP2 is widely expressed in many organs and tissues such as pancreas, white adipose tissue, lung, spleen, brain (Pecqueur et al., 2001), while UCP3 is principally expressed in skeletal muscle, cardiac muscle, and BAT (Boss et al., 1997; Vidal-Puig et al., 1997). Finally, UCP4 (6p12.3) and UCP5 (also termed brain-specific mitochondrial carrier protein-1 or BMCP1; Xp24) are expressed predominantly in the central nervous system and at a lower level in other tissues (Yang et al., 2002; Smorodchenko et al., 2009). Unlike UCP1, the physiological role of the others UCPs still remains unclear and debated. It has been hypothesized that these proteins may provide protection from oxidative damages by preventing excessive production of mitochondrial Reactive Oxygen Species (ROS) (Negre-Salvayre et al., 1997). Several observations led to this suggestion. UCP- knockout mice have increased levels of ROS and show signs of increased oxidative damage (Arsenijevic et al, 2000; Vidal-Puig et al., 2000; Brand 2002). Moreover, activation of UCP2 and -3 by ROS leads to a mild uncoupling, and to a diminished ROS formation (Echtay et al., 2002; Brand et al., 2004), while inhibition by purine nucleotides increases the membrane potential and mitochondrial ROS production (Brand and Esteves, 2005).

The uncoupling activity of UCPs has also been linked to other more specific physiological functions (for a review see: Nübel and Ricquier, 2006; Echtay, 2007). Fatty acid oxidation (MacLellan et al., 2005), glucose-stimulated insulin secretion (Chan and Kashemsant, 2006), whole body energy balance (Bezaire et al., 2007), and apoptosis (Mattson and Kroemer, 2003) are among the processes that seem to be regulated by these proteins. Consistently, in humans, a number of genetic association studies strongly support a role for uncoupling proteins in different phenotypic traits involving alterations in cellular energy homeostasis such as obesity, diabetes and lipid-related diseases (Li et al., 2008; Salopuro et al., 2009; Jia et al., 2010 and references therein). Indeed UCPs are considered potential targets for the treatment of these diseases (Armstrong, 2008).

UCPs are also believed to modulate the rate of ageing and lifespan (Brand, 2000; Wolkow and Iser, 2006; Mookerjee et al., 2010). The ageing process is largely influenced by changes in the homeostasis of the redox state and by the progressive increase in oxidative stress. Accumulation of oxidative lesions mediated by ROS causes tissue damage and energy metabolic changes which alter some cellular functions, such as growth and homeostasis (Frisard et al., 2007). The decline of homeostatic capacity of the organism with age makes less efficient not only the response to external and internal stress but also the metabolic pathways for the storage, mobilization and use of nutrients. In human, this decreased ability to regulate energy balance contributes to the phenomenon of weight and fat loss in the elderly, with important consequences on the health status (Roberts and Rosenberg, 2006). Indeed, it is well known that body weight changes in older individuals are associated with functional impairment, frailty and mortality (Fried et al., 2001).

Experimental evidences also support a role for UCP-mediated uncoupling in extending lifespan and this has led to propose the Uncoupling-to-survive hypothesis (for reviews see: Harper et al., 2004; Wolkow and Iser, 2006; Dietrich and Horvath, 2010). Of interest, targeted expression of exogenous UCP extends lifespan of adult flies (Fridell et al., 2004, Fridell et al., 2009). Mice with high metabolism live longer and have higher uncoupling activity (Speakman et al., 2004). Transgenic and knockout mice for UCP genes show alteration in lifespan (Conti et al., 2006; Andrews and Horvath, 2009).What is more, Caldeira da Silva and colleagues (Caldeira da Silva et al., 2008) demonstrated that *in vivo* uncoupling is an effective mimic of caloric restriction (CR), the only recognized non-genetic manipulation that extends lifespan in mammals (Bishop and Guarente, 2007). Consistently, mice subjected to CR show an increased expression of UCP2 and UCP3 (Bevilacqua et al., 2005; McDonald et al., 2008).

In human, the effect of the common genetic variation of UCPs on longevity has not been investigated yet. Thus, the goal of this study was to elucidate this point by specifically examining the effects of the variability of UCP genes on survival at very old age. Our results argue for a role of uncoupling proteins in human ageing and longevity.

Materials and Methods

Samples

A total of 621 (300 men and 321 women) unrelated individuals (age range 64-105 years) participated in the present study. The subjects, born and living in Calabria (southern Italy), and whose parents and grandparents were native of the same area, were collected during several campaigns of recruitment (De Rango et al., 2010). All subjects were free of clinically manifested pathologies (cardiovascular diseases diabetes,

cancer), and gave their informed consent to use their genetic and phenotypic data for genetic studies on ageing and longevity. The analyses were carried out by dividing the sample in two gender-specific age classes obtained according to the survival function of the Italian population from 1890 onward (Passarino et al., 2006). The two "thresholds of longevity" used to define these age classes were 88 years for men and 91 years for women.

SNPs selection

Polymorphisms within the UCP genes were selected using information from NCBI dbSNP and HapMap database. SNP selection was based on allele frequency, position, and functional effects. For each gene the selected SNPs are reported in Table 1.

Genotyping

DNA was prepared from peripheral blood lymphocytes using standard techniques. Genotyping of the ten polymorphic sites was carried out using a TaqMan Real Time PCR (SNP Genotyping kit, Applied Biosystems). In all assays, the fluorescent FAM dye was used to label the wild-type allele, while the fluorescent VIC dye was used to label the other one. PCR reactions were carried out in a total volume of 5 µl contained 20 ng of genomic DNA, 2.5 µl of TaqMan Universal Master mix (concentration of 2x), 0.25 µl of Custom TaqMan SNP Genotyping Assay (concentration of 20x) containing both primers and probes. The amplification protocol (60 °C for 30 seconds and then 95 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute) was performed by using a StepOne thermal cycler (Applied Biosystems). Random regenotyping were conducted to confirm the results. Unclear genotype results were excluded from the analysis

Genetic and statistical analysis

Hardy-Weinberg equilibrium analysis for genotype distribution was carried out by a $\chi 2$ goodness-of-fit test. To test the hypothesis that genetic polymorphisms of UCP genes act on the probability of reaching advanced age, multivariate logistic regression analyses were used. To implement the logistic model, genotypes were coded as binary independent variables by assigning code 1 for a genotype (or a group of genotypes encompassing a given allele) and using 0 for the remaining ones (Passarino et al 2006). To this purpose, we first performed univariate analyses (based on χ^2 test) to evaluate the recessive/dominant effect of alleles on the probability to be part of the very old age group. Genotypes including the allele showing the highest effect on such probability were then pooled together. The result is a homozygous genotype versus all the others when an allele has a recessive effect; whereas many genotypes carrying a given allele versus the other one when the effect of that allele is dominant. Genotypes correlated with the phenotype analyzed ($p < 0.15$) were then used as independent variables for building logistic multivariate models. To this purpose, a forward stepwise approach with significance level set at 0.15 was applied. The choice of this threshold avoid to exclude important variables from the model, as recommended by Hosmer and Lemshow (2000).

Odds ratios (OR) and 95% confidence intervals (95% CI) were computed from regression parameters. A set of interaction hypotheses was also tested. All statistical analyses were performed using the Statistical Package of Social Science (SPSS for Windows, version 15.0; SPSS, Chicago, IL). The significance level for statistical tests was taken to be 0.05.

Nagelkerke index (NI) was calculated as a measure of the overall effect of the multivariate model on the total variance (Nagelkerke, 1991).

Pairwise measures of linkage disequilibrium (LD) between two loci were calculated with the Haploview program (Barrett et al., 2005). The amount of linkage disequilibrium was quantified by Lewontin's coefficient (D'). Maximum likelihood haplotype frequency estimation and haplotypes reconstruction were performed with Arlequin version 3.11 for Windows, which implements an EM algorithm and a (Bayesian) ELB algorithm (Excoffier et al., 2005). Permutation test was used to examine the overall haplotype profile difference between sample groups. OR and 95% CI were also calculated comparing each haplotype versus all others combined.

Results

Genotypic and allelic data were analyzed in the two gender-specific age groups described in Materials and Methods. The overall call rate for all ten SNPs in the whole sample analyzed was 98,71%. In each group, the observed genotype frequencies were in agreement with those expected at Hardy-Weinberg equilibrium. Table S1 (online Data Supplement) shows the distribution of the observed genotype and allele frequencies of each SNP in the investigated sample groups. Table 2 reports the genotypes and the relevant genetic models selected for the multivariate analyses.

The final regression models with the relevant Odds Ratios (ORs) are shown in Table 3. Gender-specific associations were found. Indeed, as estimated on the basis of OR, in males, UCP1 rs12502572-GG and UCP4 rs9472817-GG genotypes had significant negative effect on the probability to be part of the very old age group (OR=0.593, P=0.031 and OR=0.475, P=0.008, respectively), while UCP3 rs15763-TT and UCP3 rs1800849-T/ genotypes had a significant positive effect on this probability (OR=3.396, P=0.017 and OR= 2.197, P= 0.011, respectively). In females, only UCP3 rs1800849-T/ genotypes resulted to be positively associated to the survival at very old age (OR=2.065,

P=0.016). No significant interaction between any of these polymorphisms was observed.

From these models, in order to estimate the impact of the genetic variability on the survival at very old age the NI were also calculated. We found that the proportion of variance explained by the genetic data was higher in men (NI=0.11) than in women (NI=0.03).

To further investigate the observed associations, we performed haplotype-based analyses. First, we evaluated the degree of linkage disequilibrium (LD) between pairs of SNP loci. We found a moderate LD between rs12502572 and rs769269 in the UCP1 gene (D'=0.72). Among the four SNPs in the *UCP2*-UCP3 gene region, the rs660339, rs659366 and rs15763 were in moderate LD (see Fig. 1). The rs1800849 was in moderate LD with rs15763, but it was virtually unlinked with the others. Finally, we found a very weak LD between rs9472817 and rs10498769 in the UCP4 gene, and between rs2235800 and rs5975178 in the UCP5 gene (D' = 0.58, and 0.41 for UCP4 and UCP5, respectively). Based on these LD patterns, and using a D' cutoff equal to 0.6, haplotypes were reconstructed at the UCP1, UCP2-UCP3, and UCP3 loci. Results are summarized in Tables 4 and 5 for males and females respectively.

In males, we found that the overall frequency distribution of the UCP1 (rs12502572-rs769269) and UCP3 (rs15763-rs1800849) haplotypes differed significantly between the two age classes (global p-values 0.023 and 0.030, respectively). On the contrary, the differences in the overall haplotype frequency profile of the UCP2-UCP3 polymorphisms did not result significant. Individual haplotype frequency comparisons show that a borderline significant decrease of the most common GA haplotype (OR=0.72; P=0.05) and a related significant increase of the AG haplotype (OR=1.66; P=0.007) of the UCP1 SNP occur in the very old individuals group. In the same group,

a significant decrease of the most common C-C haplotype (OR=0.65; P=0.01) and a related significant increase of C-T haplotype of the UCP3 SNPs was also observed. However, these differences between the two age classes for the UCP1 haplotypes appeared to be attributable to the rs12502572, while for UCP3 haplotypes to the rs1800849 variation. Thus, SNP combinations directly including allele showing significant single-locus associations result in significantly different haplotype frequencies between the two age groups.

As for females, no significant association was observed for any of the haplotypes estimated except for UCP3 where substantially the same trend as in males, but less significant, were found (Table 5).

Discussion

It is widely accepted that uncoupling proteins may contribute to slow down ageing and extend lifespan either by attenuating ROS production or by controlling the body metabolic rate (Brand, 2000; Wolkow and Iser, 2006; Mookerjee et al., 2010). As discussed in the introduction, several compelling evidence from model systems support this role (Harper et al., 2004; Wolkow and Iser, 2006; Dietrich and Horvath, 2010). However, there was no clear evidence of an association between UCPs genetic variability and ageing/longevity in humans. To the best of our knowledge, this is the first association study in which simultaneous analysis of the variability of the five human uncoupling protein genes (U*CP1* to *UCP5*) in relationship to longevity has been carried out.

Our results suggest that genetic variability of different *UCP* genes affects male and female survival. In fact, we found that in males *UCP1*, *UCP3*, and *UCP4*, significantly affect individual chance to become ultranonagenarians, while in females only the *UCP3*

affects this chance. Moreover, the variance explained by the genetic data is higher in males (11%) than in females (3%). These findings, which are in line with literature data, once more show that genetic factors act on survival in a gender-specific manner, and that genetic factors exert a stronger effect in male than in female survival (Tan et al., 2001).

Since a genetic polymorphism can affect a complex trait if it (or a variant in LD) does modulate the function of the genetic product and if such a product plays a role in a physiological pathway that is important for the trait under study, the impact of UCP1, -3 and -4 variability on male chance to attain longevity confirms the importance of the uncoupling process in the modulation of the ageing process. In particular, the different localization of the proteins we found associated with longevity allows to have a glimpse of the areas where the uncoupling process plays an important role in survival at old age.

As above mentioned, UCP1 is expressed almost exclusively in BAT where it plays a major role in thermogenesis (Cannon and Nedergaard, 2004). In rodents, the decline of the UCP1-mediated thermogenesis during ageing contribute to weight gain and visceral adiposity, two phenomena that are involved in the development of age-related conditions (McDonald and Horwitz, 1999). This finding suggested a mechanism for controlling body weight and energy metabolism in adults. In fact, it has been reported that UCP1 is expressed, although at low levels, also in other cell types (Sale et al., 2007; Adams et al., 2008; Zingaretti et al., 2009), and that adrenergic-stimulated white adipocytes, in response to certain stimuli such as cold exposure**,** can acquire features of brown cells (Tiraby et al., 2003). Therefore UCP1 may be an important factor that contribute to reduce the development of age-related diseases, and so may impact the chance to reach advanced ages. We hypothesize that variations in the expression or activity of UCP1 associated with the rs12502572 SNP (which is located in intron 2)

could be important for regulating changes in body composition, energy expenditure, and cold thermoregulation occurring during ageing.

UCP3 is highly expressed in skeletal muscle, and it has been reported to have *a role* in the regulation of fatty acid metabolism, maintenance of resting metabolic rate, and protection against oxidative damage (Argyropoulos and Harper 2002; Rousset, et al., 2004; Echtay, 2007). The *UCP3* rs1800849 is a C/T SNP located at position −55 in the promoter region of the gene. It has been reported that the rs1800849-T allele increased UCP3 mRNA expression in skeletal muscle compared with the C allele (Schrauwen et al., 1999). We found that the carriers of the T allele were significantly over-represented among the very old subjects, both in males and in females. Ageing muscle, is characterized by a progressive loss of mass and a gradual increase of weakness leading to sarcopenia, which is associated to physical disability and to an increased risk to develop disorders such as atherosclerosis, type II diabetes and hypertension (Karakelides and Nair, 2005). Sarcopenia is closely linked to a decrease in resting metabolic rate as well as mitochondrial dysfunction and oxidative stress (Rossi et al., 2008). Therefore, in the contest of the proposed functions of UCP3, the physiological consequence of an increased UCP3 activity in skeletal muscle might be the slowing down in the decline in muscle performance with ageing as a result of a decreased production of ROS, an increased protection of mitochondria from lipid peroxidation, and a better metabolic efficiency.

UCP4 is mainly expressed in nervous system (Smorodchenko et al., 2009). To date the physiological function(s) of UCP4 in the brain, as well as the functions of the other two neuronal UCPs (UCP2 and UCP5), are not well understood. Neurons have a very high metabolic rate and consequently a high production of ROS. UCP4 protects neurons against metabolic and oxidative stress by increasing glucose uptake and by mediating

the shift of ATP production from mitochondrial respiration to glycolysis (Wei et al., 2009). Indeed, increased levels of UCP4 reduce ROS production and mitochondrial calcium accumulation, both of which promote neuronal dysfunction and cell death, suggesting that UCP4 function is crucial for neuronal survival. Accordingly, the expression of UCP4 has been found significantly reduced in brains of subjects affected by Alzheimer disease (de la Monte and Wands 2006). Moreover, evidences have been provided that UCP4 might play a role in the responses of neurons following nutritional and temperature changes (Liu et al., 2006). It is then likely that rs9472817 SNP (which is located in intron 8) is associated with functional variation(s) affecting the function of UCP4 and this has a consequence on the ageing nervous system, leading to the observed detrimental effect of the GG genotype.

The low impact of UCP polymorphisms on female survival at old ages may be due to age-related gender differences in fat storage and fat mobilization, as well as utilization of fat as metabolic fuel (Power and Schulkin, 2008). In this context, it is important to notice that, in contrast to old men, old women are more able to maintain body temperature when exposed to the cold, possibly due to circulating hormones. In fact, mortality during cold stress is higher in old men than in age-matched women (Macey and Schneider 1993). Therefore, males more than females could be affected by subtle differences in thermoregulation due to the genetic variability of UCP genes. On the other hand, the effect of UCP3 on both male and female survival at old age is in line with the crucial role of muscle metabolism in the elderly.

In conclusion, we found that the genetic variability of UCP genes affects human longevity. This finding is in agreement with previous data showing that the storage and the expenditure of energy have a key role in survival at old age and support the Uncoupling-to –survive hypothesis. However, due to the presence of different UCP

genes, each working in specific tissues, further analyses will be necessary to understand the specific role of the uncoupling process in different tissues and, consequently, in correlation with different kinds of metabolism and, possibly, nutrients.

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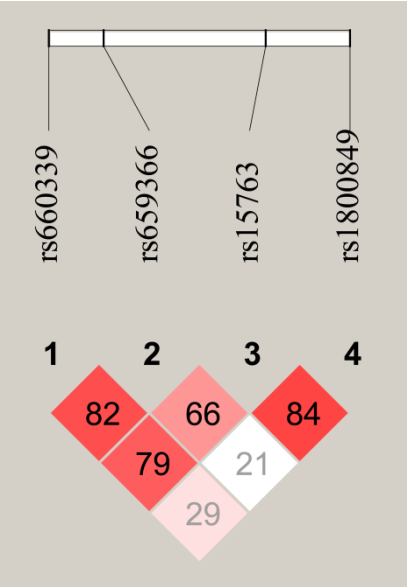

Figure 1
Schematic representation of linkage disequilibrium (D' coefficient) among the four SNPs of the *UCP2-UCP3* gene cluster.

Table 1. **Description and localization of the selected SNPs in the UCP genes.**

Gene symbol	dbSNP ID		Physical location	Function annotation *
UCP1	**rs12502572**	**A/G**	**intron 2**	
	rs7692469	**A/G**	**5'near gene**	
UCP2	**rs660339**	**C/T**	**exon 4**	**Ala55Val**
	rs659366	**G/A**	**5'near gene**	**-866 G/A**
UCP3	**rs15763**	**C/T**	**3'UTR**	
	rs1800849	**C/T**	**5'near gene**	**-55 C/T**
UCP4	**rs9472817**	**C/G**	**intron 8**	
	rs10498769	**C/G**	**5'UTR**	
UCP5	**rs2235800**	**A/T**	**intron 7**	
	rs5975178	**C/T**	**5'near gene**	

*** provided only for coding SNP (amino acid residues for two alleles) and for SNPs in the 5' flanking region (the nucleotide positions relative to the transcription start sites in the promoter regions are indicated)**

Table 2. Variables included in the final regression model.

Locus	Model in males*	χ^2	P-value	Model in females*	χ^2	P-value
UCP1 rs12502572	GG vs AG+AA	5.43	0.020	AA vs GA+GG	3.15	0.076
UCP1 rs7692469	AA vs AG+GG	2.20	0.138			
UCP2 rs660339	CC vs CT+TT	2.84	0.092	CC vs CT+TT	4.29	0.038
UCP3 rs15763	TT vs CC+CT	5.31	0.021			
UCP3 rs1800849	TT + CT vs CC	5.41	0.020	CT + TT vs CC	5.60	0.018
UCP4 rs9472817	GG vs CC+CG	8.21	0.004	GG vs CC+CG	2.47	0.116
UCP5 rs2235800	T vs A	2.49	0.115			
UCP5 rs5975178	T vs C	1.96	0.150	CC vs TT+CT	2.70	0.100

*Genotypes on the right are used as reference categories in the regression model

Table 3. Final regression models with estimated OR and p values for included variables.

Variable	OR	95% CI	P-value
Comparison in male			
UCP1 rs12502572	*0.593*	*0.368-0.954*	*0.031*
UCP3 rs15763	**3.396**	**1.239-9.310**	**0.017**
UCP3 rs1800849	**2.197**	**1.202-4.015**	**0.011**
UCP4 rs9472817	*0.475*	*0.275-0.822*	*0.008*
Comparison in female			
UCP3 rs1800849	**2.065**	**1.143-3.731**	**0.016**

CI = confidence interval. ORs were obtained directly from the equations included in the models. OR lower (in italics) and higher (in bold) than 1 indicates negative and positive effects, respectively, on the probability to be assigned to the very old age group.

Table 4 . Haplotype distribution in males

Gene				Haplotype	Old	Very old	Global P-value	OR (95% CI)	P-value
UCP1	rs12502572	rs7692469							
	G	A		GA	209	167	0.02	**0.72 (0.51-1.00)**	**0.05**
	A	G		AG	65	86		**1.66 (1.15-2.41)**	**0.007**
	A	A		AA	20	21		1.17 (0.62-2.21)	0.62
	G	G		GG	20	10		0.54 (0.25-1.17)	0.12
UCP2-UCP3	rs660339	rs659366	rs15763						
	C	G	C	CGC	188	157	0.10	0.83 (0.60-1.15)	0.26
	T	A	T	TAT	56	61		1.26 (0.84-1.89)	0.26
	T	A	C	TAC	20	22		1.23 (0.66-2.31)	0.51
	C	A	C	CAC	18	5		0.29 (0.11-0.80)	0.02
	T	G	C	TGC	14	19		1.54 (0.76-3.12)	0.24
	C	G	T	CGT	10	9		0.99 (0.40-2.48)	0.99
	T	G	T	TGT	8	9		1.25 (0.48-3.29)	0.65
	C	A	T	CAT	0	2		—	
UCP3	rs15763	rs1800849							
	C	C		CC	216	167	0.02	**0.65 (0.46-0.91)**	**0.01**
	T	C		TC	74	80		1.27 (0.88-1.84)	0.20
	C	T		CT	24	36		**1.75 (1.02-3.02)**	**0.04**
	T	T		TT	0	1		—	

Table 5. Haplotype distribution in females.

Gene				Haplotype	Old	Very old	Global P-value	OR (95% CI)	P-value
UCP1	rs12502572	rs7692469							
	G	A		GA	275	110	0.67	1.03 (0.72-1.46)	0.88
	A	G		AG	133	47		0.86 (0.58-1.26)	0.43
	A	A		AA	28	12		1.09 (0.54-2.19)	0.81
	G	G		GG	24	13		1.40 (0.70-2.81)	0.35
UCP2-UCP3	rs660339	rs659366	rs15763						
	C	G	C	CGC	295	109	0.39	0.84 (0.59-1.19)	0.32
	T	A	T	TAT	82	37		1.18 (0.76-1.81)	0.46
	T	A	C	TAC	32	17		1.38 (0.75-2.55)	0.31
	C	A	C	CAC	10	0		0.12 (0.01-2.02)	0.14
	T	G	C	TGC	18	10		1.43 (0.65-3.15)	0.38
	T	G	T	TGT	11	5		1.15 (0.40-3.37)	0.79
	C	G	T	CGT	9	3		0.84 (0.22-3.14)	0.80
	C	A	T	CAT	3	1		—	
UCP3	rs15763	rs1800849							
	C	C		CC	318	112	0.10	**0.71 (0.50-1.02)**	**0.07**
	T	C		TC	104	46		1.16 (0.78-1.73)	0.47
	C	T		CT	37	24		**1.74 (1.01-2.99)**	**0.05**
	T	T		TT	1	0		—	

Table S1. Absolute (Abs) and relative (Rel) genotypic and allelic frequences ± standard errors (E.S.) of UCP gene polymorphisms in the gender specific age-groups.

dbSNP ID	Male				Female			
	≤ 88 (N=158)		> 88 (N=142)		≤ 91 (N=230)		> 91 (N=91)	
	Abs.	(Rel. ± E.S.)	Abs.	(Rel. ± E.S.)	Abs.	(Rel. ± E.S.)	Abs.	(Rel. ± E.S.)
***UCP1* - rs12502572**								
G/G	83	0.53±0.04	56	0.39±0.04	96	0.42±0.03	37	0.41±0.05
G/A	63	0.40±0.04	65	0.46±0.04	107	0.46±0.03	49	0.54±0.05
A/A	11	0.07±0.02	21	0.15±0.03	27	0.12±0.02	5	0.05±0.02
G	229	0.73±0.03	177	0.62±0.03	299	0.65±0.02	123	0.68±0.03
A	85	0.27±0.03	107	0.38±0.03	161	0.35±0.02	59	0.32±0.03
***UCP1* - rs7692469**								
A/A	82	0.52±0.04	62	0.44±0.04	98	0.43±0.03	41	0.45±0.05
A/G	65	0.42±0.04	64	0.45±0.04	107	0.47±0.03	40	0.44±0.05
G/G	10	0.06±0.02	16	0.11±0.03	25	0.11±0.02	10	0.11±0.03
A	229	0.73±0.03	188	0.66±0.03	303	0.66±0.02	122	0.67±0.03
G	85	(0.27±0.03	96	0.34±0.03	157	0.34±0.02	60	0.33±0.03
***UCP2* - rs660339**								
C/C	76	0.48±0.04	55	0.39±0.04	110	0.48±0.03	32	0.35±0.05
C/T	64	0.41±0.04	63	0.44±0.04	97	0.42±0.03	49	0.54±0.05
T/T	17	0.11±0.02	24	0.17±0.03	23	0.10±0.02	10	0.11±0.03
C	216	0.69±0.03	173	0.61±0.03	317	0.69±0.02	113	0.62±0.04
T	98	0.31±0.03	111	0.39±0.03	143	0.31±0.02	69	0.38±0.04
***UCP2* - rs659366**								
G/G	76	0.49±0.04	62	0.44±0.04	114	0.50±0.03	42	0.46±0.05
G/A	69	0.43±0.04	70	0.49±0.04	105	0.45±0.03	43	0.47±0.05
A/A	13	0.08±0.02	10	0.07±0.02	11	0.04±0.01	6	0.07±0.03
G	219	0.70±0.03	194	0.68±0.03	333	0.72±0.02	127	0.70±0.03
A	93	0.30±0.03	90	0.32±0.03	127	0.28±0.02	55	0.30±0.03

***UCP3* - rs15763**								
C/C	89	0.57±0.04	76	0.54±0.04	136	0.59±0.03	51	0.56±0.05
C/T	62	0.39±0.04	51	0.36±0.04	83	0.36±0.03	34	0.37±0.05
T/T	6	0.04±0.02	15	0.10±0.03	11	0.05±0.01	6	0.07±0.03
C	240	0.76±0.02	203	0.71±0.03	355	0.77±0.02	136	0.75±0.03
T	74	0.24±0.02	81	0.29±0.03	105	0.23±0.02	46	0.25±0.03
***UCP3* - rs1800849**								
C/C	134	0.85±0.03	106	0.74±0.04	196	0.85±0.02	67	0.74±0.05
C/T	22	0.14±0.03	35	0.25±0.04	30	0.13±0.02	24	0.26±0.05
T/T	1	0.01±0.01	1	0.01±0.01	4	0.02±0.01	/	/
C	290	0.92±0.02	247	0.87±0.02	422	0.92±0.01	158	0.87±0.02
T	24	0.08±0.02	37	0.13±0.02	38	0.08±0.01	24	0.13±0.02
***UCP4* - rs9472817**								
C/C	36	0.23±0.03	32	0.23±0.04	56	0.24±0.03	27	0.30±0.05
C/G	69	0.44±0.04	83	0.58±0.04	115	0.50±0.03	48	0.53±0.05
G/G	53	0.33±0.04	27	0.19±0.03	59	0.26±0.03	16	0.17±0.04
C	141	0.45±0.03	147	0.52±0.03	227	0.49±0.02	102	0.56±0.04
G	175	0.55±0.03	137	0.48±0.03	233	0.51±0.02	80	0.44±0.04
***UCP4* - rs10498769**								
C/C	108	0.69±0.04	96	0.68±0.04	135	0.60±0.03	59	0.65±0.05
C/G	46	0.29±0.04	43	0.30±0.04	80	0.36±0.03	30	0.33±0.05
G/G	3	0.02±0.01	3	0.02±0.01	8	0.04±0.01	2	0.02±0.01
C	262	0.83±0.02	235	0.83±0.02	350	0.78±0.02	148	0.81±0.03
G	52	0.17±0.02	49	0.17±0.0	96	0.22±0.02	34	0.19±0.03
***UCP5* - rs2235800**								
					79	0.34±0.03	33	0.36±0.05
					123	0.54±0.03	43	0.47±0.05
					28	0.12±0.01	15	0.17±0.04
T	102	0.65±0.04	79	0.56±0.04	281	0.61±0.02	109	0.60±0.04
A	56	0.35±0.04	63	0.44±0.04	179	0.39±0.02	73	0.40±0.04

***UCP5* - rs5975178**					60	0.26±0.03	30	0.33±0.05
					113	0.49±0.03	46	0.51±0.05
					57	0.25±0.03	15	0.16±0.04
T	84	0.53±0.04	64	0.45±0.04	233	0.51±0.02	106	0.58±0.04
C	74	0.47±0.04	78	0.55±0.04	227	0.49±0.02	76	0.42±0.04

No significant departure from Hardy-Weinberg equilibrium (HWE) was observed in controls. Numbers may not add up to 100% of subjects due to genotyping failure.

5. Conclusive remarks

In my thesis I reported the investigation of UCP genes variability in human aging and longevity.

Studying *UCP1* gene emerged that haplotypic variants in the 5'-enhancer modulates survival, probably by affecting the levels of the protein. Of interest, also for *UCP3* gene a role in aging process was found. In particular *UCP3* variability impacts hand grip strength, one of the most important landmark of human aging. This correlation could have a protective role, in fact a more efficient uncoupling process has a beneficial effect on the aging muscle by slowing down its age related decay. Furthermore, by analyzing the variability of all *UCP* genes in human aging *UCP1*, *UCP3* and *UCP4* analyzed polymorphisms change during the age evidencing a role of these genes in human aging and longevity.

On the whole, these results suggest that the *UCPs* gene variability modulates survival at old ages in a gender-specific way, and, even if this is the first study of *UCPs* and human aging and longevity, these findings is supported from a variety of experimental data in model organisms. Moreover, as *UCPs* are differently expressed in various organs and tissues, it is possible that the variability of different *UCPs* may affect the senescence of different tissues with important effects on the overall aging process and then to have an important role in human longevity.

6. Appendix

During my PhD appointment I also participated to two projects on genetic variations influencing aging and longevity. The results of these a investigations have led to two publications.

In particular, the first paper titled "Somatic Point Mutations in mtDNA Control Region Are Influenced by Genetic Background and Associated with Healthy Aging: A GEHA Study" and is published on Plos One. This work indicates that: i) mtDNA Control Region heteroplasmy is genetically controlled; ii) it is beneficial for longevity; iii) it runs in families of long lived subjects.

The second paper titled "Association of a common LAMA5 variant with anthropometric and metabolic traits in an Italian cohort of healthy elderly subjects" and is published on Experimental Gerontology. Laminins are large heterotrimeric glycoproteins found in basement membranes where they play an essential role in cell-matrix adhesion, migration, growth, and differentiation of various cell types. The aim of this work was to investigate the effect of LAMA5 rs659822 on anthropometric traits (lipid profile and fasting glucose levels) in an Italian cohort of healthy elderly subjects. In conclusion, this study provides evidence that LAMA5 rs659822 regulates anthropometric and metabolic traits in elderly people motivating future investigation to elucidate whether LAMA5 rs659822 is the casual variant and the mechanisms behind the observed genetic associations.

6.1. Somatic Point Mutations in mtDNA Control Region Are Influenced by Genetic Background and Associated with Healthy Aging: A GEHA Study (published on Plos One)

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PLoS one

Somatic Point Mutations in mtDNA Control Region Are Influenced by Genetic Background and Associated with Healthy Aging: A GEHA Study

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Abstract

Tissue specific somatic mutations occurring in the mtDNA control region have been proposed to provide a survival advantage. Data on twins and on relatives of long-lived subjects suggested that the occurrence/accumulation of these mutations may be genetically influenced. To further investigate control region somatic heteroplasmy in the elderly, we analyzed the segment surrounding the nt 150 position (previously reported as specific of Leukocytes) in various types of leukocytes obtained from 195 ultra-nonagenarians sib-pairs of Italian or Finnish origin collected in the frame of the GEHA Project. We found a significant correlation of the mtDNA control region heteroplasmy between sibs, confirming a genetic influence on this phenomenon. Furthermore, many subjects showed heteroplasmy due to mutations different from the C150T transition. In these cases heteroplasmy was correlated within sibpairs in Finnish and northern Italian samples, but not in southern Italians. This suggested that the genetic contribution to control region mutations may be population specific. Finally, we observed a possible correlation between heteroplasmy and Hand Grip strength, one of the best markers of physical performance and of mortality risk in the elderly. Our study provides new evidence on the relevance of mtDNA somatic mutations in aging and longevity and confirms that the occurrence of specific point mutations in the mtDNA control region may represent a strategy for the age-related remodelling of organismal functions.

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Introduction

It has been recognized for a long time that age-related random damages to mtDNA and the consequent decrease in the respiratory chain capacity are among the major contributors to the aging process [1–4]. In fact, several studies reported that deletions and point mutations of mtDNA accumulate during aging in humans and in a wide range of organisms [5,6]. However, in the last decade different studies highlighted specific somatic mutations in the mtDNA Control Region (CR) which can reach high levels in aged individuals [7,8]. These mutations are tissue-specific and occur at mtDNA sites which are critical for replication or transcription, suggesting new important clues on the relevance of mtDNA heteroplasmic mutations in the aging process. For instance, the C150T transition that has been observed in leukocytes and fibroblasts creates a new replication site at position 149, substituting for that at 151.

Interestingly, it has been found that the CR heteroplasmic point mutations are over-represented in centenarians with respect to younger subjects in the Italian population. Data on MZ and DZ twin pairs have proposed that the heteroplasmic levels of 150C and 150T alleles were genetically controlled [9]. This hypothesis has been bolstered by analyzing centenarians' families, where we demonstrated that CR heteroplasmy in centenarians' descendants (children and nieces/nephews) are significantly higher than in age-matched controls and, moreover, they are significantly correlated in parent-offspring pairs [10]. Thus, it has been proposed that the CR somatic point mutations may represent a remodelling mechanism which would restore the replication machinery, providing a beneficial effect on longevity [9]. On the other hand, Iwata et al. [11], who analyzed the C150T mutation in leukocytes from centenarians and their offspring of an Ashkenazi Jew population, found a low incidence of the C150T mutation, but a rather high frequency of the T152C mutation. Furthermore, they found that the heteroplasmic form of the T152C transition (presumably originated from somatic events) increases with age. These findings clearly suggested population specificity on the occurrence of CR point mutations, possibly mediated by nuclear genetic variability. It may be worth noticing that population

specificity has also been observed when the effect of inherited mtDNA variability on longevity has been studied [12,13]. On the other hand no correlation was observed between inherited mtDNA variability and mtDNA CR heteroplasmy [10].

To get more insights about such complex phenomenon, in this study we took advantage of the population samples collected in the frame of the Genetics of Healthy Aging (GEHA) project. The GEHA consortium aimed to collect an unprecedented number of 90+ years old sib-pairs, from several European areas [14]. We analyzed mtDNA CR heteroplasmy in a 300 bp stretch surrounding the nt 150 position, in Lymphomonocytes and Granulocytes obtained from sib-pairs of different origin (Italy and Finland). This allowed us to further investigate the genetic control on the occurrence/accumulation of CR heteroplasmy, but also to analyze the cell specificity of CR somatic mutations. Moreover, we further analyzed the correlation between mtDNA polymorphisms and mtDNA CR heteroplasmy by defining for haplogroup classification the samples analyzed for heteroplasmy. Finally, due to the GEHA sampling strategy, we had an opportunity to verify a correlation between mtDNA heteroplasmy and health status in very old subjects (measured by means of Hand Grip strength, one the most reliable indicator of functional status in the elderly, see ref. [15] and references therein) as well as if population-specificity does exist for this phenomenon.

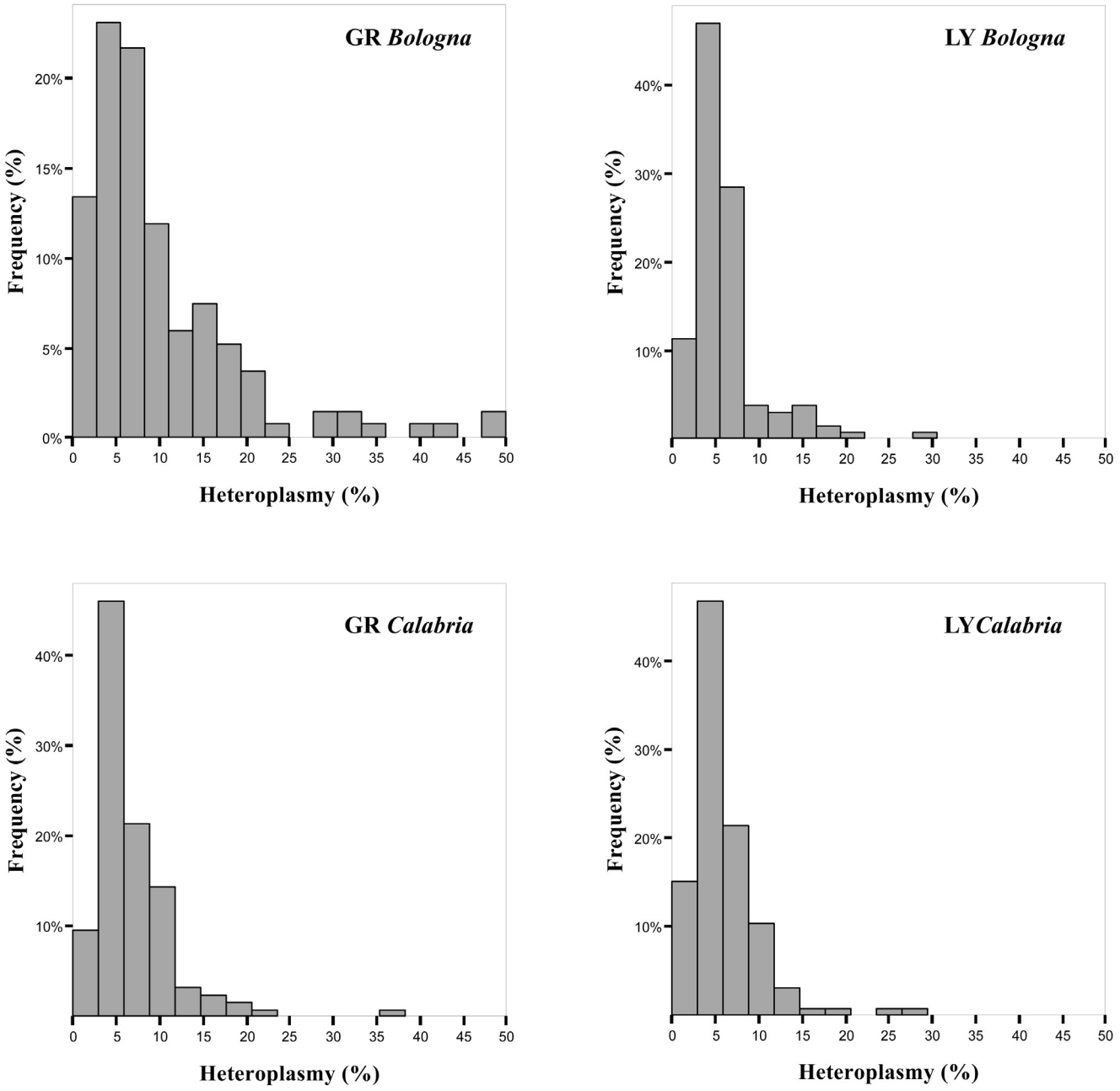

Figure 1. Heteroplasmy distribution in samples from northern and southern Italian sib-pairs. The histograms show the levels of heteroplasmy in Granulocytes (GR) and Lymphomonocytes (LY) of sib-pairs. The percentages of heteroplasmy are estimated on a DHPLC reference curve [10]. Northern Italian: Bologna; southern Italian: Calabria.
doi:10.1371/journal.pone.0013395.g001

Results

MtDNA CR heteroplasmy

Fig.1 shows the distribution of heteroplasmy levels (as defined in Materials and Methods) in Granulocytes (GR) and Lymphomonocytes (LY) from subjects collected in Bologna and Calabria. In each sample the distribution is not normal, as the majority of the subjects showed levels of heteroplasmy lower than 10%.

Linear regression analyses showed a significant correlation in heteroplasmy between siblings in both cell types (Fig.2; $p<0.05$ in all the cases). Such a correlation was further confirmed by random permutation analysis, which showed that the correlation was lost when sib-pairs were shuffled. On the other hand, the distribution of heteroplasmy in GR and LY of each subject revealed a clear somatic contribution in the occurrence or accumulation of heteroplasmy. In fact, although a strong correlation of intra-

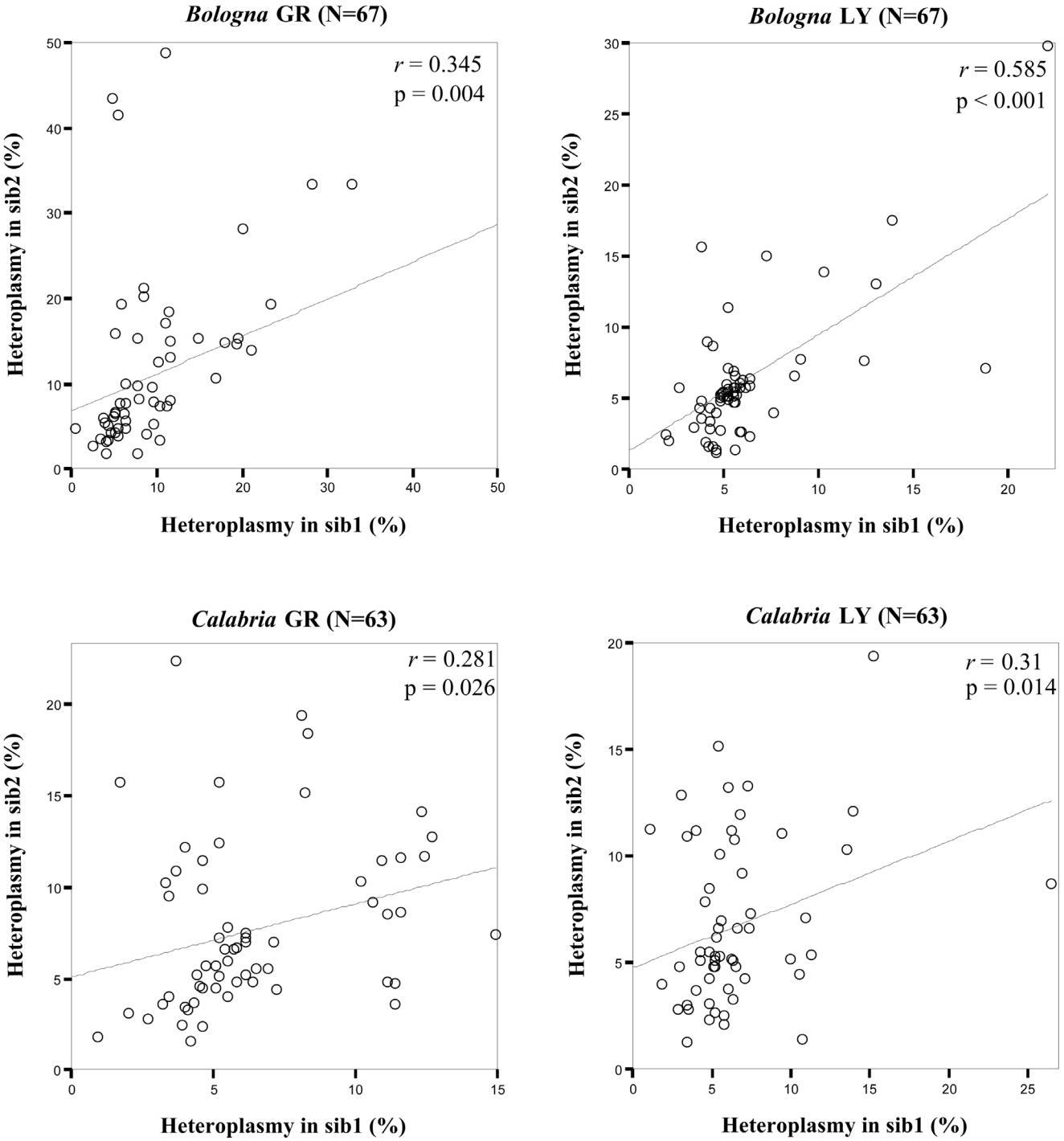

Figure 2. Correlation analyses in northern and southern Italian sib pairs. For each sample group regression lines and correlation coefficients (r) are shown. The p-values are obtained by 2-tailed Pearson test. Northern Italian: Bologna; southern Italian: Calabria.
doi:10.1371/journal.pone.0013395.g002

individual heteroplasmy was observed ($r = 0.9$, $p<0.001$) some samples were found to be heteroplasmic in one cell type only.

We then studied the heteroplasmy in a group of sib-pairs from Finland (Tampere). For this sample, DNAs from buffy coats (BC) were available. Fig. 3a shows the distribution of mtDNA CR heteroplasmy levels. Also in this case we found a significant correlation between siblings (Fig. 3b) that was confirmed by random permutation analysis.

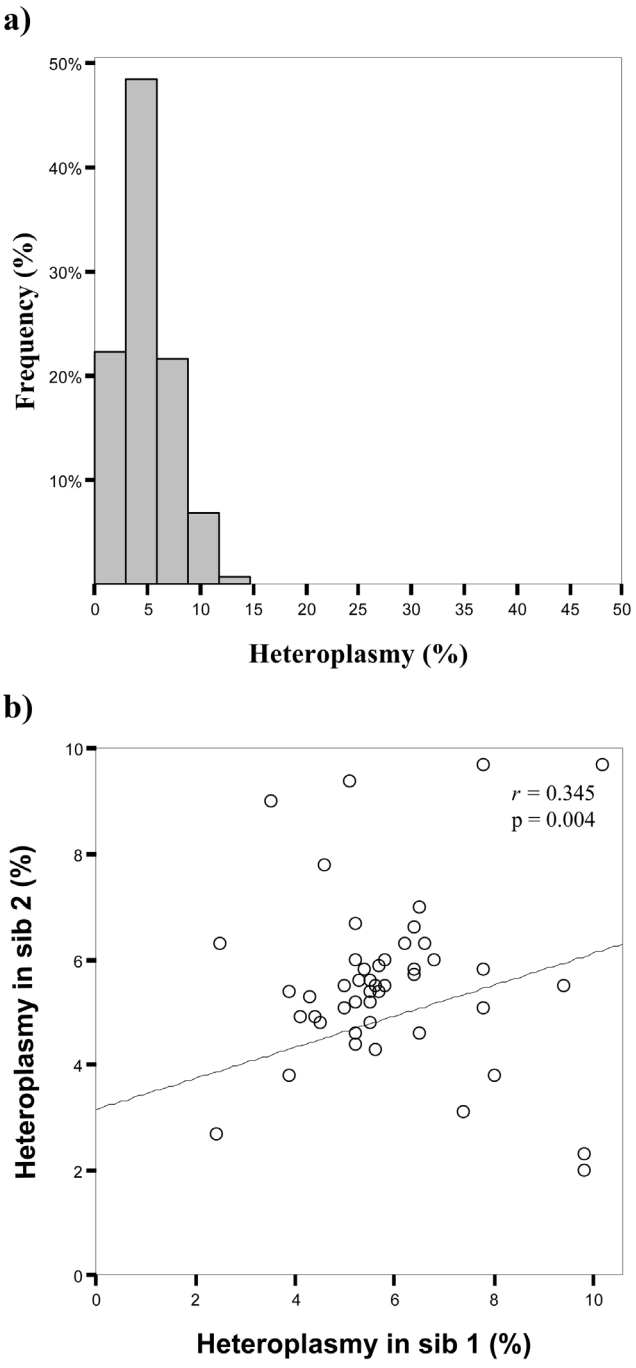

Figure 3. Heteroplasmy distribution (a) and correlation analyses (b) in Finnish sib-pairs. Percentages of heteroplasmy are estimated on a DHPLC reference curve [10]. In (b), regression analysis with correlation coefficients and p-values by 2-tailed Pearson test is shown.
doi:10.1371/journal.pone.0013395.g003

Screening of the C150T mutation

PARFAH method was used to specifically detect and quantify the heteroplasmic C150T mutation. In order to check the sensitivity of the PARFAH method, we first screened a series of samples with known levels of the heteroplasmic C150T mutation. We found that the method can reliably detect a mutant load as low as 2.5% (Fig. 4).

In Table 1 the results of the PARFAH and DHPLC analysis are summarized. We found that only a fraction of the heteroplasmic samples was heteroplasmic for the C150T mutation, while the majority of the samples were heteroplasmic for other point mutations.

Fig. 5a and 5b show the levels of the 150T allele in both GR and LY of Calabria and Bologna sib-pairs. Only 23 out of 63 and 28 out of 67 sib-pairs, respectively, exhibited the mutation in one sib at least (either in GR or in LY). A very strong correlation between sibs of each pair was observed for both cell types ($p<0.001$ both in the Calabria and Bologna samples). Although a strong intra-individual correlation was observed ($p<0.001$ both in the Calabria and Bologna samples), some subjects were found to be heteroplasmic for the C150T mutation in one cell type only, indicating a somatic contribution in the occurrence or accumulation of the mutation.

Fig. 5c shows the levels of the 150T mutation in leukocytes of Finnish sib-pairs. Only 20 out of 65 sib-pairs exhibited the mutation at least in one sib. Also for the Finnish sib-pairs, a strong correlation in mutation levels was found ($r = 0.885$, $p<0.001$).

Heteroplasmy due to mutations different than C150T

Table 1 shows that, by combining the data of DHPLC and PARFAH analyses, only a part of heteroplasmic samples was heteroplasmic for the C150T mutation. We then investigated whether the CR heteroplasmy not due to the C150T transition were correlated between sibs. Interestingly, a significant correlation was observed in Bologna and Tampere groups ($p<0.05$ in GR, LY and BC), but not in the Calabria group ($p>0.05$ in both GR and LY).

Correlation of heteroplasmy with inherited mtDNA variability

Table S1 of Supplementary Information reports for each sample the levels of heteroplasmy and haplogroup classification according to Achilli et al [16] and Ghelli et al. [17]. No correlation was observed between haplogroup classification and mtDNA CR heteroplasmy.

Correlation of heteroplasmy with physical performance

For this analysis, we considered only the elder sib from each pair. In addition, we pooled the Italian samples, for which we had data for both Lymphomonocytes and Granulocytes. We found a correlation between heteroplasmy in Lymphomonocytes and adjusted Hand Grip scores ($r = 0.147$, $p = 0.037$). Further, a marginal correlation was found ($r = 0.165$, $p = 0.075$) when we performed the same analysis on the heteroplasmy of the C150T transition. Finally, we observed a correlation ($r = 0.223$, $p = 0.027$) between the heteroplasmy in Lymphomonocytes not due to the C150T mutation and adjusted Hand Grip score. It may be worth noticing that if one uses the Bonferroni correction for multiple testing the significance threshold is 0.017, and then the observed correlations could not be considered significant.

No correlation whatsoever was observed between Hand Grip strength and the heteroplasmy level observed in the Granulocytes.

Discussion

There is increasing evidence that tissue specific heteroplasmic mutations in the mtDNA CR may favor longevity. Moreover, the

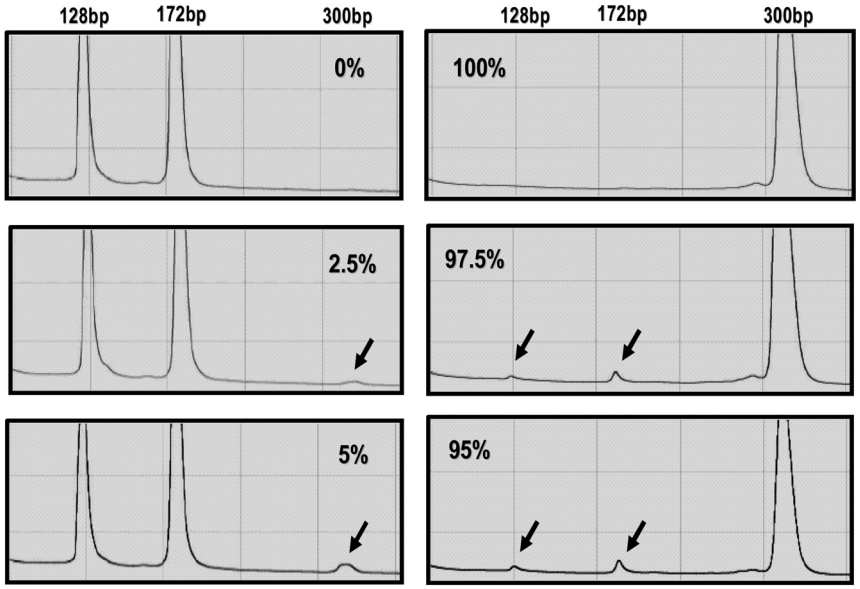

Figure 4. Chromatograms obtained for HPLC separation of digested samples with known heteroplasmy levels (PARFAH Method).
doi:10.1371/journal.pone.0013395.g004

occurrence and the accumulation of these mutations may be genetically influenced [9,10,18]. In the current study we analyzed heteroplasmy in a segment of the mtDNA CR encompassing the nt 150 position. The analysis was carried out in Granulocytes and Lymphomonocytes from one individual in ultra-nonagenarians sib-pairs that were sampled from northern and southern Europe (Finns and Italians).

The strong correlation of the total CR heteroplasmy and the C150T heteroplasmy between sibs seems to confirm that CR heteroplasmy is genetically influenced. However, we found that CR heteroplasmy not related to C150T mutation is correlated between sib-pairs only in the Finnish and northern Italian samples but not in the southern Italian sample. These results suggest that the genetic contribution to CR mutations may be population specific. Thus, it is likely that nuclear genes, environmental factors or their interaction may have different effects on the occurrence or accumulation mtDNA somatic mutations. The analysis of heteroplasmy in GR and LY showed a high level of correlation, but also revealed some subjects with quite different heteroplasmy in the two cell types. This confirms that CR heteroplasmy is a somatic event, which occurs quite late in the differentiation of these two cell types with a common origin. Thus, the intra-individual correlation may be due to the genetic control of heteroplasmy, although we can not exclude the possibility that the somatic acquisition of heteroplasmy occurs in stem cells during haematopoiesis in most cases and after their differentiation only in a few cases.

Table 1. Proportion of samples heteroplasmic for the C150T mutation.

Sample group	Heteroplasmic samples (DHPLC-based analyses)	Samples heteroplasmic for the C150T transition (PARFAH-based analyses)
Calabria **GR**	124/126 (98.4%)	25/124 (20.2%)
Calabria **LY**	121/126 (96%)	21/121 (17.3%)
Bologna **GR**	121/134 (90.3%)	29/121 (24%)
Bologna **LY**	133/134 (99.2%)	24/133 (18%)
Tampere **BC**	108/130 (83.1%)	30/108 (27.8%)

doi:10.1371/journal.pone.0013395.t001

The analysis of mtDNA haplogroups in our sample showed no correlation between CR mtDNA hetroplasmy and inherited mtDNA variability. This finding, in keeping with previous reports, confirms that the genetic factors affecting CR mtDNA hetroplasmy are likely to be nuclear genetic factors [9,10]. In addition, as somatic mutations occurring in mtDNA CR fall on different mtDNA background given by a combination of polymorphisms described by haplogroup classification, our analysis suggests that the effect CR mtDNA mutation is not modified by mtDNA variability.

The correlation between Hand Grip strength and CR heteroplasmy in Lymphomonocytes suggested by our data is quite interesting although it will need to be verified in a larger sample. If confirmed, this data will further support the positive effect of CR point somatic mutations on longevity. In fact, Hand Grip strength is one of the best markers of health status in the elderly and it has been shown to be the best single survival predictor [15,19]. It is important to point out that such a correlation was observed for Lymphomonocytes but not in the Granulocytes. In fact, we have previously proposed that the coexistence of mtDNA molecules carrying alternative replication origins due to specific CR mutations may represent an advantage in the frame of mitochondrial fusion and fission that regulates the remodeling of the mitochondrial network [20]. In fact, all the CR mutations that have been described [10,11] were related to replication origins [21]. This strategy for restoring the functionality of damaged mitochondria that have accumulated during aging is crucial for maintaining the bioenergetic efficiency of the cell and seems to play an important role in cell differentiation [22], in the regulation of apoptotic events [23] and aging [24,25]. Thus, it is evident that the presence of alternative replication origins may be more

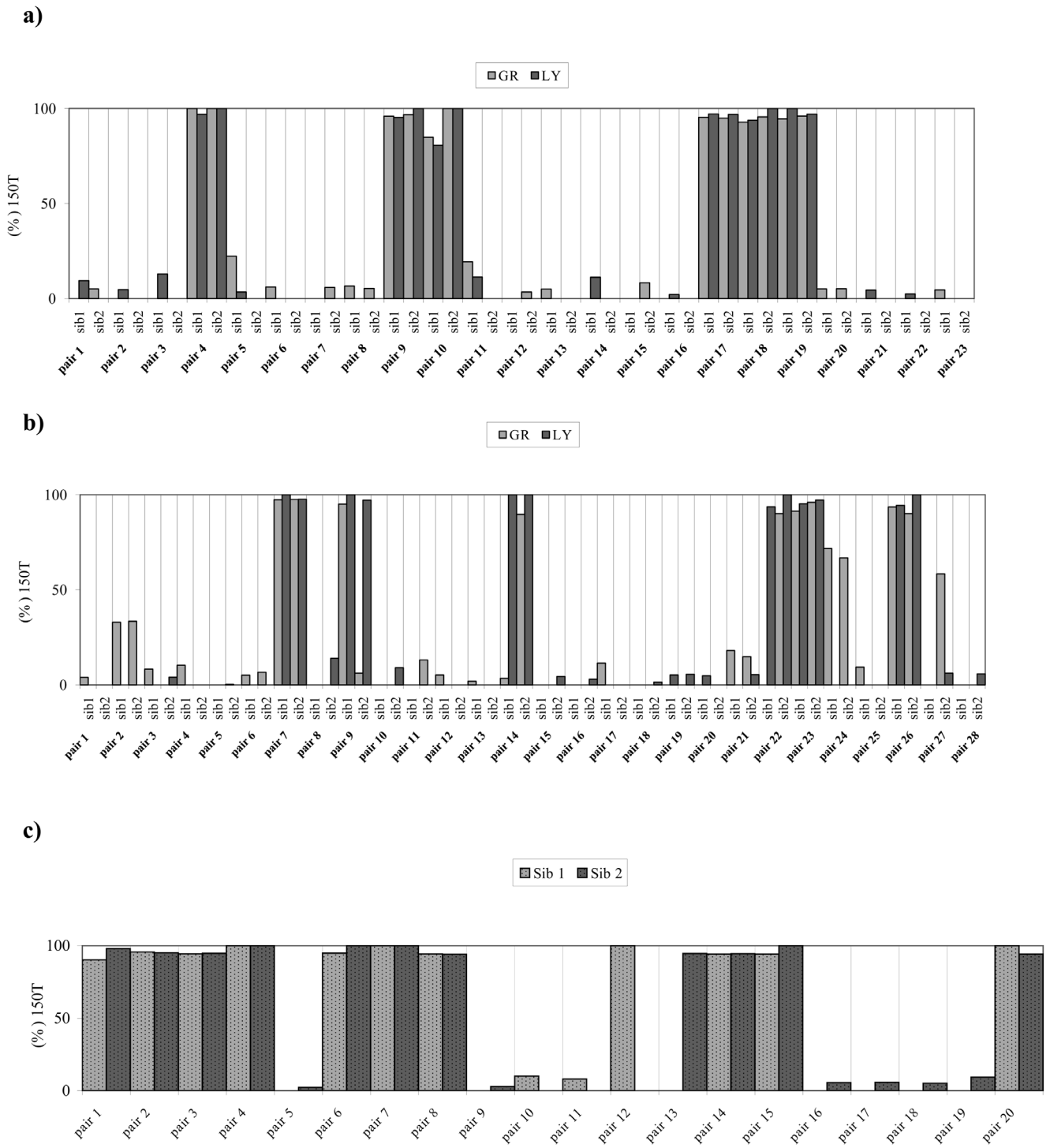

Figure 5. a) and b) Analysis of the C150T mutation in sib-pairs from *Calabria* and *Bologna* respectively. Bar graph summarizing the frequency and the distribution of the C150T in GR and LY. On the x-axis it is indicated each sib pair carrying the mutation; sib1 and sib2 refers respectively to the older and the younger sib in the pair. c) **Analysis of the C150T mutation in sib-pairs from Tampere**. Bar graph summarizing the frequency and the distribution of the C150T in Buffy Coats. On the x-axis each sib-pair carrying the mutation is indicated.
doi:10.1371/journal.pone.0013395.g005

important for Lymphomonocytes, which have an important role in response to stress and are related to frailty [26,27], than in Granulocytes, post mitotic cells living only for a few hours.

On the whole, our study has provided new important clues on the relevance of mtDNA somatic mutations, and of the consequent heteroplasmy, for aging and longevity. We confirmed the high incidence of somatic point mutations and the consequent heteroplasmy in the mtDNA CR of long-lived subjects. In addition, we showed that CR heteroplasmy is significantly correlated with physical performance in oldest olds. On the other hand, we showed that mtDNA heteroplasmy in Leukocytes may be due to the previously described C150T mutation, but also to

other mutations, the occurrence of which may be influenced in a population-specific way. In any case, the genetic control of the mtDNA CR heteroplasmy suggests that the occurrence of mtDNA somatic mutations may represent an important strategy for the age-related remodeling of organismal functions.

Materials and Methods

Ethical statement

The sampling was carried out in the frame of the GEHA project [14], according to the directions of the Ethical Board of the GEHA Project, after receiving the approval of the local Ethical committees (in particular: Ethical Committee of the University of Calabria for the samples collected in Calabria; Independent Ethical Committee of the Bologna Hospital-University for the samples collected in Bologna; Ethical Committee of the city of Tampere for the samples collected in Finland). Written informed consent was obtained from each subject.

Population samples

A total of 195 sib-pairs (390 subjects) were analyzed: 63 sib-pairs (126 subjects) were recruited in the south of Italy (Calabria), 67 sib-pairs (134 subjects) in the north of Italy (Bologna) and 65 sib-pairs (130 subjects) in Finland (Tampere). For the Italian sib-pairs we analyzed DNAs coming from two different cell-types, Granulocytes (GR) and Lymphomonocytes (LY), for a total of 520 DNA samples (252 from the south of Italy, 268 from the north of Italy); for the Finnish sib-pairs we analyzed 130 DNAs coming from Buffy Coats (BC).

Biological samples

According to the standard operating procedure the biological samples (buffy coats, Lymphomonocytes and Granulocytes) were stored locally and shipped to the National Public Health Institute (Helsinki, Finland), where DNA extraction, quality control and storage of the extracted DNA were performed. An aliquot of these DNAs was sent to us for the genetic analysis.

Quantification of the CR heteroplasmy by DHPLC

The mtDNA region under study (nt 16531-00261; 300 bp) was amplified and submitted to DHPLC [10]. The level of heteroplasmy, that is the percentage of the less frequent allele in the heteroplasmic mixture, was calculated as described by Rose et al. [10].

Quantification of the C150T mutation levels by PARFAH

Quantification of the C150T mutation was carried out by PARFAH (PCR Amplicon Restriction Fragment Analysis by HPLC) [28]. About 600–800 ng (30 µl) of PCR products were digested with 10 U of FokI endonuclease (Biolabs) at 37°C for 3 hours by adding directly the enzyme into the PCR mix. 20 µl of digested products were injected into a DNASep^{TM} column of a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic, San Jose, CA) and eluted in 0.1 M triethylammonium acetate, pH 7, with a linear acetonitrile gradient at a flow rate of 0.9 ml/min under non-denaturing conditions (over temperature 50°C). By applying this method digested fragments eluted depending on their molecular weight. FokI cleaves amplified fragments carrying the 150C allele, but does not cleave amplified fragments carrying the 150T allele. Then, the presence of the homoplasmic 150C allele was recognised by the appearance of two peaks (relevant to the fragments of 128 and 172 bp); the presence of the homoplasmic 150T allele was recognised by the appearance of one peak (relevant to the fragments of 300 bp); the presence of heteroplasmic samples was recognised by the appearance of three peaks (relevant to the fragments of 128, 172 and 300 bp) in the elution profiles. In such a case, the percentage of mutant mtDNA was calculated by measuring the percentage of peak area (WAMAKER 4.0 software, Transgenomic San Jose) related to the mutant allele.

In order to check the sensitivity of the PARFAH method, we analyzed, in three independent experiments, a series of standard mixes in which the percentage of mutant mtDNA varied from 0 to 100%.

Analysis of mtDNA inherited variability

MtDNA variability was analyzed by defining subhaplogroup of one subject of each sib pair. The analysis was carried out by sequencing the entire mtDNA control region from nucleotide position (np) 16024 to np 576. This was followed by a hierarchical survey of haplogroup and sub-haplogroup diagnostic markers in the coding region [16,17].

Physical performance

Physical performance was evaluated by means of Hand grip strength. Hand grip strength was measured by using a handheld dynamometer while the subject was sitting with the arm close to his/her body. The test was repeated three times with the stronger hand; the maximum of these values was used in the analyses. The scores obtained were subsequently adjusted for sex, age and height as these parameters turned out to be significantly correlated with Hand Grip strength.

Statistical analyses

The two-tailed Pearson test was used to perform correlation analyses. The linear correlation coefficient (r) measures the strength and the direction of a linear relationship between two variables; while the p-value measures the probability of getting a correlation as large as the observed value by random chance, when the true correlation is zero. The p-value is computed by transforming the correlation to create a t statistic having n-2 degrees of freedom, where n is the number of data pairs.

Permutation procedures were used to verify if the observed correlation in mtDNA CR heteroplasmy in sib-pairs was due to the kinship between siblings or to their concordant age.

All statistical analyses were performed by using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). A significance level of $\alpha = 0.05$ was chosen in all the tests.

Supporting Information

Table S1 Phylogenetic classification of the samples analyzed according to the haplogroup classification in comparison with the levels of heteroplasmy.
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Author Contributions

Conceived and designed the experiments: GR CF GP. Performed the experiments: GR SD PC FS. Analyzed the data: GR KM CF GP. Contributed reagents/materials/analysis tools: ACB AH. Wrote the paper: GR GP.

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6.2. Association of a common LAMA5 variant with anthropometric and metabolic traits in an Italian cohort of healthy elderly subjects (published on Experimental Gerontology)

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Association of a common *LAMA5* variant with anthropometric and metabolic traits in an Italian cohort of healthy elderly subjects

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ABSTRACT

Laminins are large heterotrimeric glycoproteins found in basement membranes where they play an essential role in cell-matrix adhesion, migration, growth, and differentiation of various cell types. Previous work reported that a genetic variant located within the intron 1 of *LAMA5* (rs659822) was associated with anthropometric traits and HDL-cholesterol levels in a cohort of premenopausal women. The present study aimed to investigate the effect of *LAMA5* rs659822 on anthropometric traits, lipid profile, and fasting glucose levels in an Italian cohort of 667 healthy elderly subjects (aged 64–107 years). We also tested for association between these traits and the single nucleotide polymorphism (SNP) rs13043313, which was previously shown to control variation in *LAMA5* transcript abundance in the liver of Caucasians. In age- and gender-adjusted linear regression analyses, we did not find association of rs13043313 with any of the traits. However, under an additive model, the minor C-allele of *LAMA5* rs659822 was associated with shorter stature ($p = 0.007$) and higher fasting glucose levels ($p = 0.02$). Moreover, subjects homozygous for the C-allele showed on average 6% and 10% lower total cholesterol ($p = 0.034$) and LDL-cholesterol ($p = 0.016$) levels, respectively, than those carrying at least one T allele, assuming a recessive model. Finally, in analyses stratified by age groups (age range 64–89 and 90–107 years), we found that the C-allele was additively associated with increased body weight ($p = 0.018$) in the age group 64–89 years, whereas no association was found in the age group 90–107 years. In conclusion, this study provides evidence that *LAMA5* rs659822 regulates anthropometric and metabolic traits in elderly people. Future studies are warranted to replicate these findings in independent and larger populations and to investigate whether rs659822 is the causal variant responsible for the observed associations.

1. Introduction

Basement membranes (BM) are sheets of specialized extracellular matrix (ECM) that surrounds epithelial, endothelial, muscle, fat, and Schwann cells. The major components of the basement membranes are laminins, a family of heterotrimeric glycoproteins consisting of three different chains (α, β, and γ) (Durbeej, 2010). In mammals, different combinations of five α, four β, and three γ chains can assemble into 18 diverse laminins that have a tissue-specific distribution (Durbeej, 2010). For example, the only laminin isoform present in the basement membrane of the human pancreatic islet cells is laminin-511 (composed of α5, β1 and γ1 chains) (Otonkoski et al., 2008). Through direct interaction with other extracellular matrix proteins and cell surface receptors, laminins mediate cell-matrix adhesion and therefore regulate migration, growth, proliferation, and differentiation of various cell types (Colognato and Yurchenco, 2000). Mutation analyses of the laminin genes in humans and functional studies in laminin mouse models have demonstrated that they have an essential role in mammalian embryonic development and organogenesis (Miner, 2008; Durbeej, 2010).

The laminin α5 chain, which is found in laminin-511 and laminin-521 (α5, β2, and γ1 chains), is encoded by the *LAMA5* gene that maps on chromosome 20q13.2–q13.3 (Durkin et al., 1997), within a region linked to inter-individual differences in body fat (Lembertas et al., 1997), serum lipid profile (Soro et al., 2002; Li et al., 2005), and susceptibility to type-2 diabetes (T2DM) (Lillioja and Wilton, 2009). By performing quantitative genetic studies in the fruit fly *Drosophila melanogaster* and a population-based study in a human cohort, De Luca et al. (2008) recently identified *LAMA5* as a potential candidate

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gene influencing body composition traits. They showed that Caucasian premenopausal women who were homozygous for the less frequent C-allele of the *LAMA5* rs659822 polymorphism had shorter stature and lower body weight, total fat mass, and lean tissue mass than those carrying at least one T allele (De Luca et al., 2008). The association of rs659822 with body weight and lean tissue mass was also observed in African-American women from the same population (De Luca et al., 2008); however, the effect of rs659822 on these traits in the African-American women had opposite direction, which suggests its context dependence with respect to other genes and/or environmental factors. In the same study, *LAMA5* rs659822 was also associated with HDL-cholesterol (HDL-C) levels in Caucasian women (De Luca et al., 2008).

Remodeling of the biochemical composition of ECM plays an important role in aging processes (Labat-Robert, 2003; Candiello et al., 2010). This observation led us to investigate the genetic effect of *LAMA5* rs659822 on anthropometric traits, serum lipids, and fasting glucose in an Italian cohort of healthy elderly subjects. We also tested for association between these traits and SNP rs13043313, which lies 16 kb upstream of the transcription start site of the *LAMA5* gene (http://uswest.ensembl.org). Previously, Schadt et al. (2008) reported a significant association between rs13043313 and variation in *LAMA5* transcript abundance in the liver of Caucasian subjects ($P = 2.61 \times 10^{-15}$), we therefore reasoned that rs13043313 might be a causal variant.

2. Materials and methods

2.1. Study subjects

The study was carried out in a cohort of 667 (358 women and 309 men) unrelated subjects (aged 64–107 years) who were enrolled during a recruitment campaign that started January 2002 in Calabria. Details of the recruitment process were reported in (Bellizzi et al., 2005). All subjects included in the present study were in fairly good health and did not manifest any major age-related pathology (e.g. cancer, T2DM, and cardiovascular diseases). Study participants, their parents, and grandparents were all born in Calabria as ascertained from population registers. A written informed consent was obtained from all participants before enrolling in the study.

2.2. Phenotypic measurements and genotyping

Height and weight were measured while subjects were dressed in light indoor clothes and without shoes. Blood samples were withdrawn after 12-h overnight fast and biochemical measurements were performed at the Italian National Research Centre on Ageing (Cosenza) using standard protocols as described elsewhere (Garasto et al., 2003).

The genotypes of rs659822 and rs13043313 were determined by TaqMan Real-Time allelic discrimination method (SNP Genotyping kit, Applied Biosystems). Random re-genotyping of the samples was conducted to confirm the results. Unclear genotype calls were not included in the analysis.

2.3. Statistical analysis

Hardy–Weinberg equilibrium (HWE), allelic frequencies, and D' linkage disequilibrium coefficients were assessed using Haploview v3.2 (Barrett et al., 2005). Linear regression models were used to test the association of each SNP with trait variation adjusted for age, gender, and appropriate potential confounding variables (see Table 1), assuming additive, dominant and recessive models (Lettre et al., 2007). The age variable was dichotomized, dividing the subjects in two age groups, based on survival curves previously constructed using Italian demographic mortality data (Passarino et al., 2006). The coding of this variable was dependent on gender. Specifically, men were indicated as being in the older group when they were above the age of 88, while women were only indicated as being in the older group if they were above 91 (Passarino et al., 2006). Analyses were

Table 1
Anthropometric and metabolic characteristics of the entire study cohort stratified according to rs659822 or rs13043313 genotypes.

	C/C	C/T	T/T	p[a]
rs659822				
n Male/female	24/21	115/146	170/191	
Age (years)	86.2 ± 0.6	86.2 ± 0.7	86.7 ± 1.7	
logBMI (kg/m^2)	.23 ± 0.02	3.22 ± 0.01	3.21 ± 0.01	0.311
Height (cm)[b]	154.9 ± 0.7	156.1 ± 0.3	157.3 ± 0.4	0.007
Weight (kg)[c]	63.9 ± 1.0	62.9 ± 0.5	61.9 ± 0.5	0.108
logFasting glucose (mg/dl)[d]	4.68 ± 0.03	4.64 ± 0.01	4.60 ± 0.01	0.020
logTriglycerides (mg/dl)[d]	4.72 ± 0.04	4.72 ± 0.02	4.72 ± 0.02	0.955
Total cholesterol (mg/dl)[e]	193.8 ± 3.8	197.2 ± 1.9	200.6 ± 1.9	0.150, 0.034[f]
HDL-C (mg/dl)[e]	4.02 ± 0.02	4.02 ± 0.011	4.03 ± 0.01	0.724
LDL-C (mg/dl)[e]	113.0 ± 3.4	116.0 ± 1.7	119.0 ± 1.7	0.156, 0.016[f]
rs13043313				
n Male/female	17/24	137/148	154/182	
Age (years)	87.4 ± 1.8	86.3 ± 0.7	86.3 ± 0.6	
logBMI (kg/m^2)	3.23 ± 0.02	3.22 ± 0.01	3.21 ± 0.01	0.497
Height (cm)[b]	156.0 ± 0.7	156.5 ± 0.3	157.0 ± 0.4	0.271
Weight (kg)[c]	63.2 ± 1.0	62.7 ± 0.5	62.2 ± 0.5	0.439
logFasting glucose (mg/dl)[d]	4.67 ± 0.02	4.64 ± 0.01	4.61 ± 0.01	0.084
logTriglycerides (mg/dl)[d]	4.72 ± 0.04	4.72 ± 0.02	4.73 ± 0.02	0.935
Total cholesterol (mg/dl)[e]	195.2 ± 3.8	197.7 ± 1.8	200.1 ± 2.0	0.309
HDL-C (mg/dl)[e]	4.02 ± 0.02	4.03 ± 0.01	4.03 ± 0.01	0.945
LDL-C (mg/dl)[e]	114.0 ± 3.4	116.4 ± 1.6	119.0 ± 1.8	0.242

Data represent means ± SE. BMI: body mass index. HDL-C: HDL-cholesterol. LDL-C: LDL-cholesterol. BMI, fasting glucose, and triglyceride levels were $\log_{10}$ transformed to fulfill the assumption of normality.

[a] p values represent the significance of the comparison among genotypes. p values without superscript were calculated assuming additive models.
[b] Adjusted for gender and age.
[c] Adjusted for gender, age, and height.
[d] Adjusted for gender, age, and logBMI.
[e] Adjusted for gender, age, logBMI, and logTriglycerides.
[f] p values were calculated assuming a recessive model.

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performed using SAS 9.1 software (SAS Institute, Cary, NC). Haplotype analyses were performed using program haplo.glm from the package HAPLO.STATS version 1.2.1 (Schaid et al. 2002) in R programming language (http://cran.r-project.org/bin/windows/base/).

3. Results

The minor C-allele frequencies of rs659822 and rs13043313 in our cohort were 0.263 and 0.277, respectively. All genotype groups were in HWE ($P>0.05$) and the genotyping efficiencies were 99.9% for rs659822 and 99.3% for rs13043313. Our analysis showed that *LAMA5* rs659822 is in LD with rs13043313 ($D' = 0.69$), which is consistent with data from the HapMap Project in the CEU population (http://hapmap.ncbi.nlm.nih.gov/). The estimated frequencies of the TT, TC, CT, and CC haplotypes were 0.665, 0.072, 0.054, and 0.204, respectively.

The means (and standard errors) of the anthropometric and metabolic variables of the study subjects stratified according to rs659822 or rs13043313 genotypes are shown in Table 1. Contrary to our prediction, there was no association of SNP rs13043313 with any of the phenotypic traits in the analysis pooled across age and gender (Table 1). However, we found a significant association between *LAMA5* rs659822 and height assuming a model of additive effect (Table 1). Each copy of the minor C-allele reduced height by 1.18 cm (95% confidence interval (CI) 0.32–2.04) in our cohort of elderly subjects, which is an effect size similar to the one previously seen in Caucasian premenopausal women (~2.3 cm difference; $p = 0.02$) (De Luca et al., 2008). Assuming the additive model, we also observed an association between *LAMA5* rs659822 and fasting glucose levels (Table 1), with each copy of the minor C-allele increasing the log of fasting glucose levels by 3.89% (95% CI 0.61%–7.28%). Finally, when a recessive model of inheritance was applied, we found that individuals homozygous for the C-allele had on average 6% and 10% less total cholesterol (CC: 187.4 ± standard error (SE) 5.58 mg/dl; TC + TT: 199.6 ± 1.99 mg/dl) and LDL-C (CC: 105.8 ± 5.0 mg/dl; TC + TT: 118.2 ± 1.36 mg/dl), respectively, than those carrying at least one T allele (Table 1). Pairwise haplotype-based association analyses between rs659822 and rs13043313 did not increase the power of these associations (Table 2).

Previous work has suggested that the effect of a given allele may change in the cell microenvironment of long-lived people (Conboy and Rando, 2005; Passarino et al., 2006). Because our cohort included nonagenarians and centenarians, we next included into the models an interaction term between rs659822 and age group. Our results showed a significant rs659822-by-age group interaction term for body weight under the additive (interaction $p = 0.052$) and dominant (interaction $p = 0.042$) models, suggesting that the effect of SNP rs659822 on these traits was not homogeneous across the two age groups. Thus, we decided to analyze the data separately for the age range 64–89 and 90–107 years. While no difference was observed in the group of people aged 90–107 years, we found that the C-allele was additively associated with increased body weight in the age group 64–89 years (Fig. 1), with each C-allele increasing body weight by 2.38 kg (95% CI 0.42–4.34). The lack of association in the very old people is not due to differences in genotype frequencies since no statistical differences were observed between the two age groups ($\chi^2 = 0.617$, $p = 0.735$).

Table 2
p-values for haplotype effects.

Trait	CC	CT	TC
logBMI (kg/m^2)	0.3677	0.6454	0.9841
Height (cm)[a]	0.0603	0.0453	0.6975
Weight (kg)[b]	0.1988	0.4076	0.7021
logFasting glucose (mg/dl)[c]	0.0927	0.0136	0.1139
logTriglycerides (mg/dl)[c]	0.9484	0.7452	0.8480
Total cholesterol (mg/dl)[d]	0.3023	0.1721	0.5352
HDL-C (mg/dl)[d]	0.8889	0.6182	0.9968
LDL-C (mg/dl)[d]	0.2308	0.3421	0.5974

p-values in this table are for the test of the effect of the given haplotype compared to the reference haplotype (TT). HDL-C: HDL-cholesterol. LDL-C: LDL-cholesterol. BMI, fasting glucose, and triglyceride levels were $\log_{10}$ transformed to fulfill the assumption of normality.

[a] Adjusted for gender and age.
[b] Adjusted for gender, age, and height.
[c] Adjusted for gender, age, and logBMI.
[d] Adjusted for gender, age, logBMI, and logTriglycerides.

4. Discussion

The present study provides evidence that the minor C-allele of *LAMA5* rs659822 is associated with reduced adult height in Caucasian elderly people as previously observed in a cohort of premenopausal women (De Luca et al., 2008). Whether rs659822, which is located within the intron 1 of *LAMA5*, is the causal variant and the mechanism behind its effect on height remain to be elucidated. Studies in mice have previously shown that embryos of animals deficient in the laminin α5 chain exhibit several developmental abnormalities, including dysmorphogenesis of the placental labyrinth (Miner 2008), which is necessary for gas exchange and the transfer of nutrition between the maternal and fetal circulation (Cross et al., 2002), and die late in embryogenesis (Miner, 2008). These studies have also revealed that laminin α5 plays an important role in kidney development by controlling glomerulogenesis (Miner, 2008). Furthermore, three-week old mice homozygous for a hypomorphic mutation in the *Lama5* gene have been reported to display loss of renal function and smaller size than controls (Miner, 2008). In humans, body size at birth, which is influenced by both the genetically predisposed fetus and intrauterine environmental factors, is positively correlated with adult height (Sorensen et al. 1999; Tuvemo et al., 1999). Thus, it is possible that genetic variation in *LAMA5* may affect fetal growth and organ development, and ultimately influence organ functions and body size in adult life, by modulating placental development and function. This hypothesis deserves further investigation considering that fetal and placental size and the consequent infant size at birth have been shown to be important predictors for the development of obesity, hypertension, T2DM, and cardiovascular disease later in life (Barker, 1990). Consistently, short stature has been associated with increased risk of coronary heart disease, T2DM, and glucose intolerance (Paajanen et al., 2010; Lawlor et al., 2002). Our study

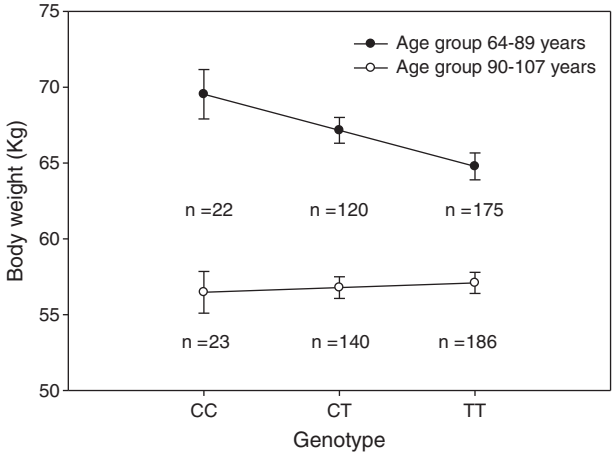

Fig. 1. Body weight by rs659822 genotype in two groups aged 64–89 and 90–107 years. Data represent means ± SE. In the age group 64–89 years, there was a significant difference among rs659822 genotypes in body weight ($p = 0.018$; adjusted for height and gender). In the age group 90–107 years, there was no difference ($p = 0.706$). n = number of subjects within each genotype.

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cohort was composed of healthy elderly subjects, but our finding of a significant association of rs659822 C-allele with fasting hyperglycemia in the entire cohort and body weight in the subjects aged 64–89 years is consistent with the hypothesis of a potential role of *LAMA5* in fetal programming. A study examining the effect of *LAMA5* rs659822 on anthropometric traits in pre-pubertal children is currently underway to provide additional evidence. Nevertheless, given the crucial role that ECM remodeling plays in skeletal development (Ortega et al., 2004) as well as in white adipose tissue development and growth (Mariman and Wang, 2010), we cannot exclude the possibility that other mechanisms, not necessarily exclusive, may underlie our findings.

One important result of the present study is that despite the genetic predisposition to gain weight conferred by the C-allele of *LAMA5* rs659822 seen in subjects 64–89 years of age, individuals homozygous for this allele who have reached 90 or more years did not show any significant difference in body weight compared to those with at least one T allele (Fig. 1). Because data on body composition measurements were not available, we cannot say at this point whether the effect of rs659822 C-allele on body weight is driven by increased fat mass and/or reduced lean mass. Whatever the tissue, one possible explanation for our results is that the effect of the *LAMA5* rs659822 allele on body weight may be influenced by the remodeling of the physiological function that occurs with aging (Barbieri et al., 2009; Capri et al., 2008). Furthermore, since energy intake rate has been shown to alter the expression of extracellular matrix genes in mice adipose tissue (Higami et al., 2006), the effect of the allele could also be modulated by differences in dietary patterns between the two age groups. Our finding might have important implications for previous work by Paolisso et al. (1995) who reported that healthy centenarians are less prone to the changes in body composition associated with aging and future studies are warranted to further investigate this matter.

In this study we did not replicate the association between *LAMA5* rs659822 and HDL-C previously observed in premenopausal women (De Luca et al., 2008). The failure to replicate the association in a population with distinct genetic background and different age might reflect the complexity of the genetic processes that determine variation in this trait, including potential epistatic interactions between genetic variation in *LAMA5* and other gene polymorphisms (Greene et al., 2009). On the other hand, we found that subjects homozygous for the C-allele had lower total cholesterol and LDL-C than those with at least one T allele, further suggesting that *LAMA5* plays a role in cholesterol metabolism. In this regard, it is noteworthy to mention that Idaghdour et al. (2010) have recently provided evidence of a regulatory relationship at the transcriptional level between *LAMA5* and *OSBPL2* genes. *OSBPL2* encodes the oxysterol binding protein-like 2 (ORP2) which belongs to a family of intracellular lipid receptors (Fairn and McMaster, 2008). Previous work reported that ORP2 regulates cholesterol homeostasis by controlling cholesterol trafficking and the endocytic pathway involved in the transport of LDL-derived cholesterol into the cell (Hynynen et al., 2005). Hence, it is possible that *OSBPL2* represents the molecular link between *LAMA5* and cholesterol metabolism.

In conclusion, our data provides evidence of a role of *LAMA5* in regulating anthropometric and metabolic traits in elderly people. It also shows that the effect of *LAMA5* rs659822 on body weight seen in subjects aged 65–89 years is blunted in the group of people who have reached 90 or more years. These findings motivate future work to investigate whether *LAMA5* rs659822 is the casual variant and the mechanisms behind the observed genetic associations.

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