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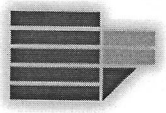

UNIVERSITA' DELLA CALABRIA

Dipartimento di Biologia, Ecologia e Scienze della Terra

PhD program in:

Life Sciences

CICLE

XXX

Genetic variability of amino acid transporters: study of the influence on physical decline and human survival

Scientific Disciplinary Sector B-18

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List of abbreviations

4E-BP1	eIF4E-binding protein
AA	amino acid
AAT	amino acid transporter
ACC	acetil CoA carboxilasi
ACE	angiotensin converting enzyme
ACEi	angiotensin converting enzyme inhibitor
AD	Alzheimer's disease
ADL	activity daily living
AdoNet	S-adenosil-l-methionine
ADP	adenosine diphosphate
AGE	advanced glycation end
AKT	protein kinase B
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
AP1	activator protein 1
APOE	apolipoprotein E
ASCT2	alanine, serine, cysteine-preferring transporter 2
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
BCAA	branched chain amino acid
BH4	tetrahydrobiopterin
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CASTOR1	Cytosolic Arginine Sensor For MTORC1 Subunit 1
CASTOR2	Cytosolic Arginine Sensor For MTORC1 Subunit 2
CAT	cationic amino acid transporter
CD98	cluster of differentiation 98
CES-D	center for epidemiologic studies depression scale
CESS	conditionally essential amino acid
CHD	coronary heart disease
CNS	central nervous system

CO	carbon monoxide
CR	calorie restriction
CREB	cyclic adenosine monophosphate response element-binding protein
CRP	c-reactive protein
CVD	cardio vascular disease
DNA	deoxy-ribonucleic acid
DR	dietary restriction
EAA	essential amino acid
ECM	extracellular matrix
eIF2α	eukaryotic translation initiation factor 2-alpha
eIF4E	eukaryotic translation initiation factor 4E
EWGSOP	European Working Group on Sarcopenia in Older People
FGF21	fibroblast growth factor 21
FOXA	forkhead box A
FOXO	forkhead box O
GABA	gamma-Aminobutyric acid
GAP	GTPase activating protein
GCK	glucokinase
GCN2	general control nonderepressible 2
GEF	guanine nucleotide exchange factor
GH	growth hormone
GHR	growth hormone receptor
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GPR120	G-protein coupled receptor 120
GPR40	G-protein coupled receptor 40
GWAS	genome-wide association study
H2O2	hydrogen peroxide
HDL-C	high density lipoprotein cholesterol
HG	handgrip
HMB	beta-hydroxy-beta-methylbutyrate
HSF	heat shock transcription factor
IF	intermitted fasting
IGF-1	insuline-like growth factor 1
IL-6	interleukine-6

ILP	insulin like peptide
IPC	insulin producing cell
IR	insulin resistance
IRP	immune risk profile
LAMPTM4b	lysosomal associated transmembrane protein 4b
LAT1	large neutral amino acid transporter 1
LAT2	large neutral amino acid transporter 2
LAT3	large neutral amino acid transporter 3
LAT4	large neutral amino acid transporter 4
LDL	low density lipoprotein
LPA	lipoprotein A
mGluR1	glutamate selective G-protein couple receptor 1
mGluR4	glutamate selective G-protein couple receptor 4
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mRNA	messenger ribonucleic acid
mSIN1	mammalian stress-activated map kinase-interacting protein 1
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAG	N-acetylglucosamine
NEAA	non essential amino acid
NO	nitrogen monoxide
NRF2	nuclear factor erythroid 2–related factor 2
PASK	PAS domain containing serine/threonine kinase
PAT1	H+/amino acid transporter 1
PAT4	H+/amino acid transporter 4
PD	Parkinson’s disease
PEPCK	phosphoenolpiruvato carboxi kinase
PI3K	phospho-inositolo 3 kinase
PIEKK	phospho-inositolo 3 kinase related kinase
PR	protein restriction
PRAS40	proline-rich Akt substrate of 40 kDa
PROTOR	protein observed with RICTOR

PUFA	poly-unsatured fatty acids
Rab1A	Ras-related protein Rab-1A
Rag GTPase	Ras-related GTPase
RAG-A	Ras-related GTP binding A
RAG-B	Ras-related GTP binding B
RAG-C	Ras-related GTP binding C
RAG-D	Ras-related GTP binding D
RAPTOR	regulatory-associated protein of mTOR
RAS	renin-angiotensin system
RDA	recommended daily allowance
RE	resistance exercise
RHEB	Ras homolog enriched in brain
RHEB1	Ras homolog enriched in brain
RICTOR	rapamycin-insensitive companion of mTOR
ROS	reactive oxygen species
Sdc	syndecan
SDC4	syndecan 4
SLC	solute carrier
SNAT2	sodium-coupled neutral amino acid transporter 2
SNAT3	sodium-coupled neutral amino acid transporter 3
SNAT7	sodium-coupled neutral amino acid transporter 7
SNAT9	sodium-coupled neutral amino acid transporter 9
Snf1	sucrose non-fermenting 1 protein kinase
SNP	single nucleotide polymorphism
T1R	taste receptor 1
T2R	taste receptor 2
TNF-α	tumor necrosis factor alpha
TOR	target of rapamycin
tRNA	transfer ribonucleic acid
v-ATPase	vacuolar proton-translocating ATPase
WT	walking time

Sommario.

Il presente progetto di ricerca si inserisce nell'ambito della tematica generale di ricerca del laboratorio di Genetica dell'Università della Calabria, relativa allo studio della comprensione dei meccanismi intrinseci (genetici ed epigenetici) ed estrinseci (ambientali e culturali) che modulano la qualità dell'invecchiamento in Calabria, volto alla identificazione di parametri in grado di definire la fragilità (frailty) degli anziani e la loro tendenza alla disabilità, alla morbidità e alla mortalità. Tra le principali componenti della frailty vi è la sarcopenia, una sindrome correlata all'età caratterizzata dalla perdita progressiva generalizzata della massa muscolare e della forza fisica, che di solito comincia a manifestarsi intorno ai 50 anni di età e colpisce, approssimativamente, il 30% degli adulti sopra i 60 anni ed il 50% degli anziani sopra gli 80 anni. La sarcopenia ha un profondo impatto negativo sulla qualità della vita del soggetto, in quanto, oltre alla perdita di massa e forza muscolare, essa è associata ad una riduzione della capacità aerobica, dello stato metabolico e ad una perdita progressiva della densità ossea, che correla con un più alto rischio di fratture e con la perdita graduale dell'indipendenza fisica del soggetto anziano. La natura multifattoriale della sarcopenia, a cui concorrono sia fattori genetici che ambientali e comportamentali, come uno stile di vita sedentario e una dieta a basso contenuto proteico, oltre a interazioni gene-ambiente, determina una grande variabilità interindividuale. Mentre gli effetti dei fattori ambientali sono stati ampiamente studiati, solo recentemente si è iniziato ad affrontare lo studio delle influenze genetiche specifiche sulla performance muscolare, che possono spiegare la variabilità interindividuale. Uno dei maggiori determinanti della sarcopenia è il declino con l'età della capacità di rispondere a stimoli anabolici. Tra i principali fattori anabolici per la sintesi proteica muscolare vi è la disponibilità di aminoacidi (AA), e nello specifico di aminoacidi essenziali (EAA), i quali rivestono un ruolo importante nella risposta del muscolo, agendo da veri e propri sensori della concentrazione degli AA, principalmente attraverso l'attivazione del pathway dell'mTORC1 (il principale sistema di sensing dei nutrienti). Dati di letteratura suggeriscono che un ruolo importante nella risposta del muscolo alla disponibilità di AA è rivestito dai trasportatori degli aminoacidi (AAT). In particolare, alcuni studi hanno dimostrato che l'espressione di alcuni di essi viene influenzata in maniera età dipendente dalla supplementazione degli EAA unitamente all'attività fisica. Tuttavia pochi sono i dati sul ruolo dei trasportatori nell'invecchiamento umano e nella disabilità ad esso associata.
L'ipotesi del presente studio è che i polimorfismi a singolo nucleotide (SNPs) appartenenti a geni codificanti gli AAT, in particolare quelli con un ruolo chiave nei meccanismi che attivano

la segnalazione mTORC1 in risposta ai livelli di AA, possano avere un impatto sulla regolazione del metabolismo proteico nel muscolo e il declino fisico associato all'età.

Nello specifico, sono stati analizzati geni codificanti per trasportatori coinvolti nel trasporto di leucina (*SLC3A2*/CD98, *SLC7A5*/LAT1, *SLC7A8*/LAT2, *SLC43A1*/LAT3), glutammina (*SLC1A5*/ASCT2, *SLC38A2*/SNAT2, *SLC38A3*/SNAT3, *SLC38A7*/SNAT7) e trasportatori lisosomiali (*SLC36A*/PAT1 e *SLC38A9*/SNAT9).

Sulla base di diversi parametri, tra i quali localizzazione genomica, frequenza nelle popolazioni, ruolo funzionale, sono stati selezionati 58 SNPs, capaci di marcare la variabilità genetica nelle regioni geniche analizzate. Tali marcatori sono stati quindi genotipizzati, mediante tecniche differenti in relazione alle specifiche caratteristiche di ogni singolo polimorfismo, in una coorte di soggetti di età compresa tra i 50 e 108 anni per un totale di 729 individui.

In un sotto-campione di età compresa tra i 50 e gli 89 anni è stata quindi verificata la correlazione tra le varianti polimorfiche studiate e parametri relativi alla performance fisica, quali la forza nella stretta della mano (HG, handgrip), la capacità di svolgere attività di vita quotidiana (ADL, activity daily living) e il tempo di camminata (WT, walking time), marcatori affidabili dello stato funzionale ed efficaci predittori di disabilità e mortalità negli anziani, verificando altresì l'associazione con la sopravvivenza, attraverso uno studio longitudinale che ha considerato un tempo di follow-up di 120 mesi. Inoltre, al fine di verificare se le varianti analizzate avessero un'influenza anche sulla probabilità di raggiungere età molto avanzate, si è studiata la correlazione con la longevità analizzando, mediante approccio trasversale, un campione di soggetti di età compresa tra i 90 e i 108 anni.

Le analisi condotte nel presente lavoro forniscono evidenze a supporto dell'ipotesi che la variabilità dei geni per AAT sia in grado di modulare la performance fisica dopo la quinta decade di vita, influenzando la qualità dell'invecchiamento e la sopravvivenza, probabilmente attraverso la regolazione dell'attività di mTORC1. Inoltre, analisi di interazione tra geni diversi ed effetti sulla sopravvivenza evidenziano l'esistenza di un'influenza complessa di tali trasportatori su tratti età correlati e longevità umana, le cui conseguenze fenotipiche potrebbero essere determinate da effetti pleiotropici legati all'età.

In conclusione, il presente studio rappresenta il primo tentativo di correlare la variabilità genetica degli AAT allo stato fisico in età avanzata. Sebbene le associazioni qui riportate non possono essere considerate esaustive da un punto di vista clinico, il lavoro qui descritto contribuisce alla comprensione della suscettibilità di questo tratto correlato all'età e supportano studi futuri sul ruolo degli AAT nell'invecchiamento e nella qualità della vita umana.

Summary.

This research project is part of a general research topic of the Genetics laboratory of University of Calabria, aimed to improve the understanding of intrinsic (genetic and epigenetic) and extrinsic (environmental and cultural) mechanisms modulating the quality of aging in Calabria, through the identification of functional parameters which can define the frailty in the elderly and their tendency to disability, morbidity and mortality. Among the major components of the frailty, sarcopenia is defined as an age-related syndrome, characterized by a generalized progressive loss of muscle mass and physical force, which usually starts around 50 years of age and affects approximately 30% of adults over the age of 60 and 50% of the elderly over the age of 80. Sarcopenia has a profound negative impact on the individual quality of life, as it is associated with a reduction in aerobic capacity, metabolic status and progressive loss of bone density, in addition to mass loss and muscle strength, correlating with a higher risk of fractures and a gradual loss of physical independence of the elderly subject. The multifactorial nature of sarcopenia, which is influenced by both genetic and environmental-behavioural factors, such as a sedentary lifestyle and a low protein diet, as well as by gene-environment interactions, leads to a huge inter-individual variability. While the effects of environmental factors have been extensively studied, only recently they started to study the specific genetic influences on muscle performance, which may explain inter-individual variability. One of the major determinants of sarcopenia is the age-related decline of the ability to respond to anabolic stimuli. Among the major anabolic factors for muscle protein synthesis there is the availability of AAs, and specifically of EAAs, which play an important role in muscle response, acting as real sensors for the concentration of AAs, activation of the mTORC1 pathway (the most important nutrient sensing system). Literature data suggest that an important role in muscle response to AA availability is played by AATs. In particular, some studies have shown that the expression of some of them is influenced in an age-dependent manner by the EAAs supplementation, together with physical activity. However, few data can be retrieved on the role of AATs in human aging and related disability.

The hypothesis of the present study was that SNPs belonging to genes coding for AAT, especially those with a key role in the mechanisms activating mTORC1 signalling in response to AA levels, may have an impact on regulating protein metabolism in muscle and age-related physical decline.

Specifically, genes encoding for carriers involved in the transport of leucine (*SLC3A2*/CD98, *SLC7A5*/LAT1, *SLC7A8*/LAT2, *SLC43A1*/LAT3), glutamine (*SLC1A5*/ASCT2, *SLC38A2*/

SNAT2, *SLC38A3*/SNAT3 and *SLC38A7*/SNAT7) and lysosomal transporters (*SLC36A*/PAT1 and *SLC38A9*/SNAT9) were analysed. 58 SNPs, able of marking the genetic variability in the analysed gene regions, were selected, taking into account several parameters, including genomic location, frequency in populations and functional role. These markers were then genotyped by different genotyping techniques, in relation to the specific characteristics of each polymorphism, in a cohort of subjects aged 50 to 108, for a total of 729 individuals.

In a sub-sample aged between 50 and 89, the correlation between the polymorphic variants studied and physical performance parameters was investigated; parameters chosen were HG, the ADLs and WT, reliable markers of the functional status and effective predictors of disability and mortality in the elderly. In the same sample, association with survival was verified through a longitudinal study, by considering a 120-months follow-up time. In addition, in order to verify whether the variants analysed could influence the likelihood of reaching very advanced ages, correlation with longevity was studied by cross-sectional approach in a sample of subjects aged between 90 and 108.

The analysis carried out in this study provides evidences to support the hypothesis that AAT genes variability can modulate physical performance after the fifth decade of life, affecting the quality of aging and survival, probably through the regulation of mTORC1 activity. In addition, interaction analysis between different genes and effects on survival support a complex influence of such transporters on age-related traits and human longevity, whose phenotypic consequences could be determined by pleiotropic age-related effects.

In conclusion, this study was the first attempting to correlate the genetic variability of AAT to the physical status at advanced ages. Although the associations here reported cannot be considered exhaustive from a clinical point of view, they contribute to our understanding of the susceptibility of age-related functional status, supporting future studies on the role of AATs in quality of aging and human lifespan.

Chapter 1

Aging: why and how!

Aging is one of the most normal and healthy aspects of life. As we age, the body accumulates the changes for which an octogenarian is more likely to die than a twenty year old. Despite the inevitability, some aspects of how and why we age are still little known in human biology (Jin, 2010). Through thousands of years, man has been fascinated by the possibility of living longer and has always looked for different ways to extend the passing of time. According to the Italian history of the last 10 years, it is possible to observe fewer births and more aged people. The last Istat report showed that in Italy the population in old age (individuals aged 65 and over) exceeds 13.5 million and accounted for 22.3% of the total population (Figure 1).

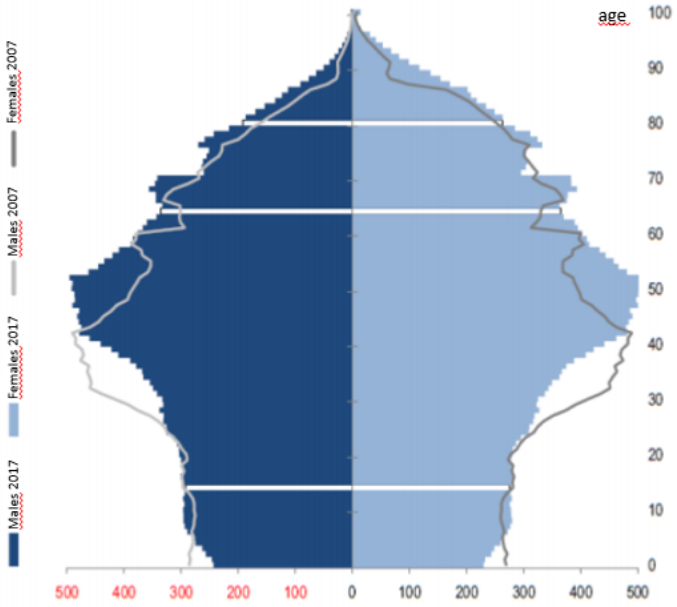

Figure 1: Pyramid of the population resident in Italy, the comparison between 2007 and 2017 (Report Istat 2017).

Furthermore, as showed in Table 1, ten years ago the nineties constituted only 0.8% of the total, but since then they have increased steadily, reaching 1.2%.

2007		2017
11.7 millions 20.1% population	**More than 65 years old**	13.5 millions 22.3% population
466.700 0.8% population	**More than 90 years old**	727.000 1.2% population
10.386 0.02% population	**Centenarians**	17.000 0.03% population

Table 1: Aged population at January 1st in Italy.

This data showed the Italian situation but that can be extended to the world population. In fact, the United Nations World Population Prospects in 2015 revealed Europe as the most aged continent, having 24% of the population aged over 60 years. For this reason, the World Health Organization (2002) has established for several years guidelines to promote healthy aging and it is important to continue to investigate the biological mechanisms that regulate the aging process in order to prevent age related diseases and develop targeted interventions for the aging modulation.

1.1. Why: Theories of aging.

Senescence is the biological process characterized by a slow and progressive, anatomical and functional age-related decay of an organism. It is a continuous physiological process, which manifests itself in different ways from individual to individual. Given the detrimental effects of aging, it is important to explain the mechanisms involved. Several experts describe aging as a multifactorial complex process and more than 300 biological theories have been developed over the years (Weinert and Timiras, 2003; Jin, 2010).

In 1890, Weisman suggested that aging is an evolution of the species and not of the individual, because in this way you can delete the older subjects that compete with young people for resources. He also postulated the cell death as a scheduled event of the cells after a certain number of divisions, which is different among species and related to lifespan (Lipsky and King, 2015). In fact, it seems that the average lifespan within a given species is genetically programmed in one way or the other. Nevertheless, the current theories of aging differ in viewing aging as a consequence or a side effect of genetic pathways (Sergiev et al., 2015).

In 1908 Max Rubner observed that larger animals outlived smaller ones and that the larger animals have slower metabolism: from this observation he postulated the "rate of living theory", according to which the faster an organism's metabolism, the shorter is its lifespan (Rubner, 1908). In 1928, Raymond Pearl conducted a series of experiments in drosophila to corroborate Rubner's initial observation (Pearl, 1928). Some years later, Kleiber gave further strength to these observations through the so-called "mouse-to-elephant" curve: he concluded that basal metabolic rate could be predicted by taking ¾ the power of body weight (Speakman, 2005).

In 1952, Peter Medawar gave the evidence that aging could be related to DNA damage accumulation (overall mutations in genes involved in DNA repair process). He developed

the accumulation mutation theory and recognized that wild animals succumb well before attaining old age because of the environment, predators, disease or accidental death. Therefore, the natural selection selects out disadvantageous mutations expressed in early life and exerts little pressure to disembarrass organisms to mutation causing detrimental effects at older age. According to this theory, harmful genes passively accumulate from generation to generation (Lipsky and King, 2015).

In 1957, Williams introduced the theory of antagonistic pleiotropy, according to which some genes exert more than one effect and some effects might benefit early survival but cause harm later in life (Kirkwood, 2005). According to this, genes related to aging have an early positive effect and they are actively retained in the gene pool. Several studies supported this theory (Benes et al., 2001; De Benedictis et al., 2001; Bonafè et al., 2002; Lescai et al., 2009; Park et al., 2009-2010; Cirulli et al., 2011; Nebel et al., 2011; Ruiz et al., 2011). For example, genes enhancing oxidative processes may generate damaging free radicals that age an organism but offer a survival advantage in youth by generating greater muscle effectiveness. In 1961, Hayflick theorized that the human cells ability to divide is limited to approximately 50-times, after which they simply stop dividing (the Hayflick limit theory of aging) (Hayflick and Moorhead, 1961). Antagonistic pleiotropy also explains that theory: early in life, this limit may suppress tumor growth (Krtolica, 2001) but it is also the reason of cellular senescence later in life.

Some scientists postulated the psychological theories of aging: the activity theory, the continuity theory and the disengagement theory. In 1961, Robert J. Havighurst developed the activity theory, also known as normal theory of aging, lay theory of aging and implicit theory of aging: he proposed that successful aging happens when people stay active and have social interactions (Havighurst, 1961). That theory reflects how the equilibrium that an individual develops in middle age should be maintained in later years (Ebersole, 2005). The acceptance of activity theory has diminished, but nowadays it is still used as a standard to compare observed activity and life satisfaction patterns. In 1964, Benice Neugarten in support of this theory proposed that satisfaction in older age depends on active maintenance of personal relationships (Bengtson and Putney, 2009). George L. Maddox gave the first empirical description of the continuity theory of aging in 1968, and then Robert Atchley formerly proposed it in 1971. He stated that elders would maintain the same relationships, activities and behaviors as they did in their early years of life (Atchley, 1989). The disengagement theory of aging, formulated by Cumming and Henry in 1961, set "aging as an inevitable, mutual withdrawal or disengagement, resulting in decreased interaction

between the aging person and others in the social system he belongs to" (Ebersole, 2005; Cumming and Henry, 1961).

According to the neuroendocrine theory, in aging process there are programmed functional changes in neurons and associated hormones. Neuroendocrine programming are for example pubertal changes and menopause in women (Troen, 2003). In 1971, Dilman conceptualized the neuroendocrine theory of aging: he underlined the existence of a self-regulatory mechanism of homeostasis, essential in hypothalamus-pituitary-adrenal axis. An elevation of the threshold of the hypothalamus to negative feedback signaling accounts for the unfavourable age-related changes (overall, the reproductive decline) (Dilman, 1971; Dilman and Anisimov, 1979).

In 1972 Kirkwood proposed the "disposable soma theory": organisms balance the need for the repair and maintenance of somatic cells with the resources needed for successful reproduction. If the resources are not limitless, organisms support survival and reproduction, and they age because accumulated damage and incomplete repair leads to a greater susceptibility to disease and environmental stress (Harman, 2006). In particular, the most prevalent damages occurs in DNA duplication process: usually, the greatest part of that damage is repaired by the cells, but they are detrimental when accumulated in non-dividing cells (Jin, 2010).

According to programmed theory, in aging an internal biological clock sequentially switch genes on and off, regulating also growth, development and maturity. Weismann first described the programmed senescence (Weismann, 1882). Later on, Skulachev extended the theory: senescence of mammals is a deleterious program, though spread over an extended period and implemented through the formation of ROS in mitochondria (Skulachev, 2012). Moreover, Boiko asserts that senescence is an acquired program: aging itself is genetically programmed because of the formation of post-mitotic tissues, given that the cells of such tissues are non-dividing and cannot be renewed by stem cell populations (Boiko, 2007).

1.2. Evolution of the aging theories.

The modern concept of aging is inspired by the early theories but amplifies them in terms of genes, pathways and molecular mechanisms, thanks to the evolution of scientific research. The approach of "extreme phenotypes" is based on the identification of subjects with clinically relevant phenotypes to maximize the efficiency in identifying the molecular pathways and the genetic characteristics underlying such phenotypes. Genome-wide analysis

of long-lived and young individuals within a species allows to identify the genes affecting longevity, and a differentiation between relevant and non-relevant mutations. In that way, genome analysis of different species could reveal the genes involved in the lifespan process. However, the analysis of species with various lifespan poses additional challenges: the genome of different species differ so much that their comparison is infeasible. In general, the lifespan is controlled by body size and potentially can vary up to 10000-fold in organisms that differ in morphology and body size. Therefore, the best approach could be the genetically comparison among related similar-sized species with different lifespan (Sergiev et al., 2015 and references therein). For this reason, the choice of the model animal for the study of longevity becomes the main point in the search for factors involved in human survival.

Genes contribute to longevity even if the extent of genetic versus non-genetic factors contributions to aging remains poorly understood (Cournil and Kirkwood, 2001; Passarino et al., 2016). In humans, twins studies showed that the genetic contribution in aging is about 20-30% (Barzilai et al., 2012), but is not yet clear how genes contribute to these. If we consider the theory of antagonist pleiotropy, it is clear that the positive or negative effects of individual genes are not constant. For example, harmful genes like Huntington's disease genes remains in the gene pool because they may exert positive effects early in life in favour of natural selection, while the harmful effects occur after the reproductive years (gene escape natural selection) (Lipsky and King, 2015).

Consistent with the idea that the genetic control of aging is multifactorial, there is no single gene but scientists postulated that aging genes act by stopping or slowing biochemical metabolic pathways. Several studies have been conducted in different animal models in order to detect mutation leading to lifespan improvement. Among these, an emerging role was given to insulin/IGF-1 pathway: mutation in the insulin receptor in C. elegance cause a two-fold lifespan increase, which underline as this pathway is important for growth and cell division (Finch and Ruvkun, 2001). Insulin-like signaling activity and the expression of insulin-like peptides are reduced in long-lived nematodes, mice, and humans (Kenyon, 2010; Moskalev et al., 2014). A small number of genes in Drosophila melanogaster has been implicated in lifespan modification too: a significant increase in lifespan is associated with mutation in insulin/IGF-1s pathways and mutations directly affecting metabolic activity (Anisimov, 2008).

In humans, aging process is characterized by changes in body composition, insulin resistance, and physiologic declines in IGF-1, sex steroids and GH. The decrease in GH secretion with age results in a decline in IGF-1 levels, associated with heart disease,

sarcopenia, osteoporosis, and frailty. The cross-linking theory of aging, for example, postulates that aging is due to glucose binding to proteins that impairs their biological functions and is associated with connective tissue curing, renal disorders and cardiac enlargement. Not only, sugars bound to DNA cause replication errors leading to age-related disease, such as cancer or diabetes: for this reason, some endocrinologists consider diabetes as an accelerated aging process (Bjorksten and Tenhu, 1990).

With aging, there is an immune system decline, which makes the organism more susceptible to infection, cancer, Alzheimer's and cardiovascular disease. Some postulate this theory as immuno-senescence (Rozemuller et al., 2005). According to the stochastic theories, aging is the result of random changes accumulated with time and failure in body's repair mechanisms. Weisman introduced the theory of accumulated damage, known also as "wear and tear" theory: he compared organism to a machine used and damaged with time from accidents, disease and other chance (Lipsky and King, 2015). Consistent with this theory is also the impairment of regulatory pathways: for example, it promotes chronic inflammation because of the unbalance between pro- and anti-inflammatory components. According to inflamm-aging theory of Franceschi, an elevated predisposition of inflammatory disease in early age protect us from infections, but at the same time is detrimental in the elderly (Franceschi et al., 2000a,b).

Stress, as oxidative stress, speeds telomere shortening and telomere dysfunction is a characteristic of premature aging syndromes, such as Werner's syndrome (Tosato et al., 2007). Telomere shortening can also be considered as an example of antagonist pleiotropy: this process protects against the uncontrolled cell division and abnormal growth associated with cancer at the price of aging (Boccardi and Herbig, 2012).

In 1950, Denham Harman introduced the free radical theory of aging, according to which organisms age over time due to accumulation of damage from free radicals in the body, produced in mitochondria's metabolic processes (Harman, 1956). ROS, which arise both as a by-product of normal metabolism and from external sources, cause oxidative damage to proteins, DNA and lipids. In 1970, scientists proposed that mitochondria generates most of the free radicals that damage the electron transport chain enzymes (Harman, 2003; Sanz and Stefanatoz, 2008). Given that these free radicals promote aging, the idea is to limit their formation: enzymes such as superoxide dismutase can neutralize them. In foods, there are some natural non-enzymatic antioxidants, such as vitamins A and C, and some phytochemicals as carotenoids and flavonoids.

This theory has been supported by several studies, which link a lower basal metabolic rate to increased life expectancy: Giant Tortoise for example can live over 150 years, and studies

in centenarians link a decreased thyroid activity to their longevity (de Magalhães et al., 2007). At the same time, there is a great variability in animals' ratio of resting metabolic rate and total daily energy expenditure, but also in coupling between ATP production and oxidative phosphorylation, in the amount of DNA repair and of saturated fat in mitochondrial membranes, that affect maximum lifespan (Speakman et al., 2002). Furthermore, a number of species with high metabolic rate, like bats and birds, are long-lived: in 2007, a modern statistical method showed that metabolic rate does not correlate with longevity in birds or mammals (de Magalhães et al., 2007).

Gladyshev suggests that the accumulation of biological junk in the organism is responsible for cell senescence (Gladyshev, 2013). Some of the by-products formed in biochemical reactions are eliminated by excretion or enzymatic degradation. The cell division contribute to the by-products dilution: the limit of this process is related to those cells that lose the replicative capacity (such as cardiomyocytes and brain neurons in human) and accumulate metabolic waste (Sergiev et al., 2015).

1.3.How: healthy aging and age related disease.

In the last few decades, scientists witnessed a growing interest in studies on human aging and longevity, overall because of the social responsibility connected to the increase of elder population in developed countries that implies an increase of non-autonomous subjects affected by invalidating pathologies (Christensen et al., 2008; Report Istat, 2017 sources).

Several studies have immediately faced the problem to identify any effective solution against the age-related detrimental consequences evident in long lived people. Aging is considered the major risk factor for common age-related pathologies (Niccoli and Partridge, 2012). However, as supported by recent theories, there is a continuum between chronic aging diseases and aging (Kennedy et al., 2014).

An important change in aging is the loss of some irreplaceable cells, as those of the brain, heart and skeletal muscle. The loss of neurons is associated with physiological changes including circadian rhythm and altered metabolism, which contribute to emotional and mental aberrations in elderly (Hung et al., 2010). Neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD) are characterized by increased ROS levels in brain tissues and other molecular mechanisms, such as programmed cell death, mitochondrial dysfunction, glia cell activation, proteasomal malfunction, protein misfolding (Andersen, 2004).

As the brain ages, inclusion bodies and abnormal proteins and lysosomes are observed more frequently. It is still unclear whether these defects promote neurodegeneration or whether they are the consequence of such diseases, and which one is the relationship between such lesions and cognitive impairment. In AD for example, an important role is also played by the inflammatory status: in fact, the production of cytokines is upregulated and they play a dual role, since they can be protective or they can cooperate as pro-apoptotic stimuli (Richartz et al., 2005). Even the alteration of many signaling pathways may contribute to the pathogenesis of these disease, such as caloric restriction, sirtuins, mitochondrial function, TOR and insulin/IGF-1 signaling (Bishop et al., 2010).

The cardiovascular diseases (CVD) are responsible for over than 40 million deaths in Europe. The majority risk factors are diabetes, lack of physical activity, tobacco use, overweight/obesity, cholesterol and high blood pressure (these factors can be controlled), but the normal process of aging is associated with progressive deterioration in structure and function of the heart and vasculature (responsible of heart failure, hypertension, coronary heart disease) (Costa et al., 2015). In fact, the aged heart undergoes a number of functional changes that reduce its ability to respond to increased workload and, the cardiac mechanisms responsible for protection from injury become defective leading to increased dysfunction and accentuated adverse remodelling (Strait and Lakatta, 2013).

Atherosclerosis is one of the pathological basis of cardiovascular disease, extremely frequent in elderly and even more in frail elderly, but also a primary cause of mortality in western world (Libby, 2002; Heidenreich et al., 2011). Coronary heart disease (CHD) is also a leading cause of death for elderly and the main risk factors are physical inactivity, family history, obesity, dyslipidaemia, tobacco smoking, hypertension and diabetes (Roger et al., 2011). Hypertension for example is considered a compensatory mechanism for age-related vascular changes; however, as showed by trials on hypertension treatment, blood pressure control leads to reduction in clinical ends of adverse outcomes (Jackson and Wenger, 2011).

Diabetes is very common in elderly: in particular, the type 2 could be found alone or in association with the metabolic syndrome (the pro-inflammatory activity of the adipose tissue leads to IR and reduce the production of insulin by pancreatic Langerhans islet cells) (Bloomer et al., 2009). In aging, the reduced ability to maintain glucose homeostasis can lead to glucose toxicity: the type 2 diabetes mellitus, in fact, is responsible of some complication in kidneys, eyes and arteries, because of the formation of advanced glycation end (AGE) products (Kim et al., 2012; Mortuza et al., 2013; Yang et al., 2013; Liu et al., 2014). Type 2 diabetes mellitus is a complex disease with a genetic component but also due to environmental factors (Doria et al., 2008; Ahlqvist et al., 2011; Brunetti et al., 2014).

Recently, scientists were interested on the impact of this disease on protein metabolism and skeletal muscle mass, because this tissue is the most affected by diabetes given that it is responsible of glucose uptake upon insulin stimulation and the main protein reservoir of the organism (DeFronzo and Tripathy, 2009; Mitchell et al., 2016). Several data, in fact, suggested that insulin is able to influence protein metabolism in human skeletal muscle through the inhibition of protein degradation (Gelfand and Barrett, 1987; Pacy et al., 1991; Nair et al., 1995; Godil et al., 2005). A number of authors suggest that the muscle loss is not a clinical feature of type 2 diabetic subjects, and they are associated only in specific clinical contexts. The important point is the appearance of complications like chronic kidney disease and cardiovascular comorbidity, associated with increased IL-6 and myostatin levels, which favour the muscle atrophy (Doehner et al., 2012; Fülster et al., 2013).

Moreover, according to the Korean Sarcopenic Obesity study, elderly with diabetes have more risk to become sarcopenic than the healthy ones: sarcopenia in fact has been associated with insulin resistance and obesity in a particular condition called "sarcopenic obesity" (Baumgartner et al., 2004; Rolland et al., 2009; Kim et al., 2010). This is due to the endocrine function changing caused by obesity as the reduction of adiponectin, IGF-1 or testosterone (that promote protein synthesis) and an increase of myostatin, leptin IL-6 and TNFα (that enhance protein degradation) (Kob et al., 2015).

In the development of the age-related disease, the alteration of immunological profile (termed as "immunosenescence") contributes to some pathology like AD, autoimmune disease and atherosclerosis. Moreover, the incidence of these pathologies is increased with advancing age and at the same time may contribute to enhance the chronic inflammatory process (Fulop et al., 2005). Otherwise, chronic low-level inflammation has been found associated with frailty, sarcopenia and other chronic age-related diseases, maybe due to antigenic stimulation throughout life (Franceschi et al., 2000a). With age, in particular, it was observed that the T regulatory cells mediate the self-tolerance and their deletion leads to autoimmune disease. These kind of cells influence the response to infections and several studies have shown that they are increased with aging, contributing to the reduced proliferation capacity of T cells (Lages et al., 2008). Longitudinal studies have brought to the definition of "immune risk profile" (IRP) as a mortality predictor, based on several adaptive immunity parameters (the most important of these is the inverted CD4:8 ratio), informative from 65 years of age (Wikby et al., 2008; Pawelec et al., 2009).

One might expect that the genetic factors leading to common complex disease, such as AD, diabetes, cardiovascular diseases and cancer, may negatively affect lifespan and be less common in long-lived subjects compared to the younger ones. Several studies suggest that

the presence of genetic "risk factors" for some disease does not always compromise longevity (Sebastiani et al., 2012; Freudenberg-Hua et al., 2014) (Figure 2). For example, it was found that some AD susceptibility genes identified by using GWAS, did not affect human lifespan, with the exception of APOE (Shi et al., 2012). The angiotension I converting enzyme (ACE) reduces blood pressure by acting on the blood vessels through the I/D polymorphism: the D allele is correlated to hypertension and myocardial infarction (Chen et al., 2013) and, at the same time, the DD genotype carriers have a reduced risk of AD (Wang et al., 2014).

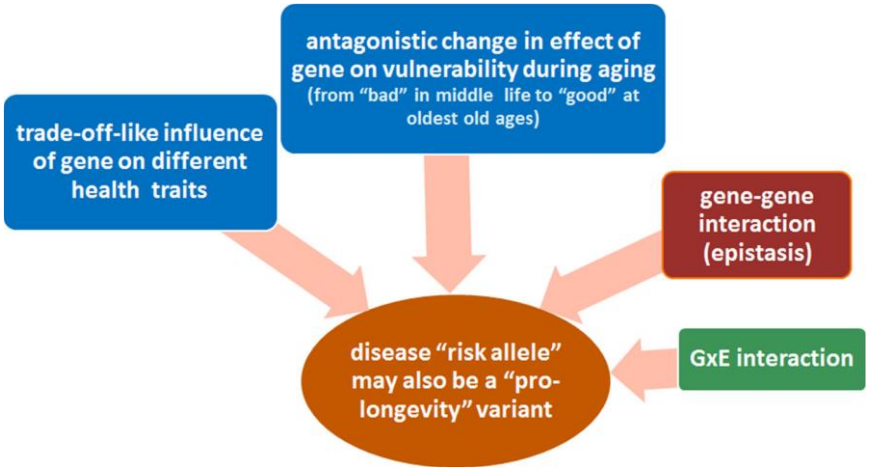

Figure 2: Genetic "risk factors" not always compromise longevity (Ukraintseva et al., 2016).

A number of studies showed an epidemiological trade-off among major complex diseases and suggested their potential impact on longevity: for example, individuals with AD may have lower risk of cancer (Akushevich et al., 2013). Lipoprotein (a) is a risk factor for CVD and shows a trade-off-like influence on major human diseases: LPA levels are risk factors for peripheral artery disease, stroke and CHD (Enkhmaa et al., 2011); however, some LPA genotypes were found to be protective against other non-CVD disorders like cancer (Sawabe et al., 2012).

Currently centenarians are considered the paradigm of the uninterrupted quest for immoratality and they have the "secret" of life. There are several population known in the world as long-lived, because they reach at least 100 years of age and more, and several scientists are interested in studying them. It was immidiately clear that aging has different effects in females and males, and there are gender-specific factors of longevity (the male/ female ratio is related to environmental and social factors (Montesanto et al., 2017).

From the Okinawa Centenarians Study, an increase of centenarians from 1975 to 2016 results (from 30 to 1000 centenarians): this could be due to an improvement of life

expectancy by generation more evident in women (87% of centenarians are females) (Willcox et al., 2017). In Japan, a study conducted on supercentenarians (110+) showed that these subjects are characterized by the marked postponement of age-related debilitation and the retention of physical independence for an extraordinarily long period (Arai et al., 2017). The Chinese Longitudinal Healthy Longevity Study reports data which refers to Han Chinese centenarians and clearly point out a lifelong much higher socioeconomic status of male centenarians compared to their female counterparts (important information to interpret health and cognitive status) (Yi et al., 2007).

Endocrine parameters and related pathways significantly affect longevity and aging. However, there is a population showing a particular phenotype and studied since 1958 by Laron and colleagues. This cohort, living in Ecuador, is characterized by homozygous mutation in GHR, people are short and they have obesity. At the same time, they live longer and cancer incidence is lower compared to the controls.

Chapter 2

Dietary manipulation and effects of nutrients on aging and age-related diseases.

The high interest of research is to understand genetic and non-genetic factors affecting aging and age-related diseases. New approach of preventive medicine is based on some studies showing that pharmacological, genetic and dietary interventions could be efficient for ameliorate age-related outcomes. In particular, recent work underlined that specific dietary interventions promote long life and healthy old age (Fontana and Partridge, 2015; Dato et al., 2016). Among these interventions promising results came from intermittent fasting and protein restriction, caloric or dietary restriction (CR, DR) that extend lifespan up to 50% (Fontana et al., 2010; Longo et al., 2015).

However, the relationship among human health, diet and longevity is complex, considering the metabolic pathways, the nutritional components, the physiologic process involved for the cell survival. It is also important to consider the interaction of multiple polymorphisms which could change how some nutrients may affect gene expressions (the so-called nutrigenomic) and how some individuals may respond to dietary interventions (the so-called nutrigenetics) (Darnton-Hill et al., 2004; Santoro et al., 2014). It is almost clear that individuals with different genotypes respond differently to diet, although little studies have analysed the genetic effects in humans to identify who can benefit from dietary manipulation. Furthermore, males and females respond very differently to pharmacological and nutritional intervention, thus more studies are needed.

2.1. Dietary strategies.

Nutrition is that process by which organisms take food for metabolic and growth processes. Nutrients are the building blocks and the energy sources for simple and complex organisms. They have the capability to regulate cell function, such as to initiate cell-signaling events and regulate gene expression in the absence of hormonal influences. A typical human diet is composed by water and fibers (important for the intestinal absorption of nutrients), macronutrients (such as lipids, carbohydrates and proteins that provide the calorie needs) and micronutrients (such as essential minerals and vitamins that support metabolic and biochemical activities). The modern diet is almost considered the responsible of nutritionally

related diseases such as obesity, metabolic syndrome, type 2 diabetes mellitus, renal failure and insufficiency, cerebrovascular accidents, heart failure, myocardial infarction, CHD, atherosclerosis and hypertension (Everitt et al., 2006; Houston, 2010; Kant, 2010; Allès et al., 2012; Rahe et al., 2014).

In elderly, it is evident a gradual decline in energy intake, in particular an imbalance between energy from carbohydrates (increased) and from fat (decreased): the low fat and protein intake is associated with mortality, while carbohydrates show a threshold effect on frail patients (Frisoni et al., 1995; de Groot and van Staveren, 2010). Currently, the scientist are trying to find the nutritive regimen followed by long-lived people, but to study the dietary intake of centenarian is very difficult because it is not practical to monitor food intake over 100-year lifespan and because of the imprecision in recording it. Furthermore, there is a heterogeneity of body mass index and nutritional status of centenarians, influenced by residence region, dietary and lifestyle factors (Hausman et al., 2011).

Longevity is governed by a complex matrix, a sort of metabolic adjustment of autonomous and non-autonomous responses, very difficult to clarify. For this reason, several studies were performed to analyse those effects to slow the aging rate and increase health span and lifespan. While genetic and pharmaceutical interventions have been widely explored in laboratory models (Baur et al., 2006; Mitchell et al., 2014), the same approaches in humans are difficult to be applied (Fontana and Partridge, 2015).

CR is considered a conservative mechanism to extend lifespan. This diet contain 25-50% less calorie compared to a normal diet, but maintain adequate levels of vitamins and minerals (overall vitamin B3, nicotinamide adenine dinucleotide phosphate or NADP, and nicotinamide adenine nucleotide or NAD). The principal molecular mechanism observed in rodents is the lower rate of tissue oxidative damage, associated with lower rate of mitochondrial free radicals and slower accumulation of DNA damage. Several studies demonstrated that DR improves health in both rats and mice (Fontana et al., 2010). Other molecular effectors include NRF2, HSF, sirtuins, AMPK, TOR, and FOXO, which upregulate cell proliferation, stress resistance, antioxidant activity, autophagy, and DNA repair through several “longevity pathways” (Guarente, 2013; Johnson et al., 2013; Burkewitz et al., 2014; Webb and Brunet, 2014) (Figure 3).

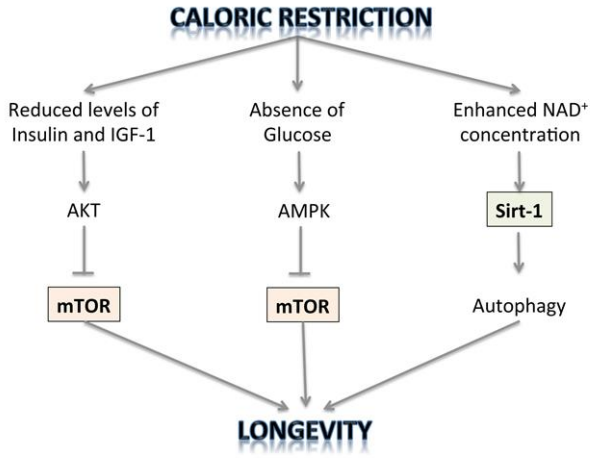

Figure 3: Some "longevity pathways" involved in CR (Mangan, 2015).

Clinical trials studies of DR in humans show significant reduction of skeletal muscle and myocardial impairment, and reduction of risk factors for metabolic diseases (Barzilai and Bartke, 2009; Cox and Mattison, 2009). Acute responses to DR involve insulin sensitivity, reduced inflammation, and protection against surgical stressors (Hine et al., 2015). However, if on one side, it is evident that DR can extend the healthy human lifespan, at the same time, there are not enough data to understand the effects on life expectancy and the beneficial effect does not appear to be universal.

Several studies have demonstrated that excess nutrient availability may be detrimental to brain function: in fact, diet not only modify the neuronal matrix, but also control insulin secretion, energy balance, glucose homeostasis and nutrient utilization in the whole body (Bishop et al., 2010). The main factors responsible of brain architecture modification are synaptic plasticity, adult neurogenesis and inflammation, which operate through paracrine, hormonal and humoral factors, as well as direct effects (Pani, 2015). Brain plasticity is important for learning and memory and a decline of this capacity is a distinctive feature of neurodegenerative disorders (Mattson, 2012). Therefore, changes in function, structure and number of synapses, as well as changes in neurons itself, are modulated by metabolic and nutritional inputs.

The new findings about the nature of effector mechanisms, the role of specific nutrients and the importance of timing in food intake have pointed the attention to less drastic dietary manipulations to improve healthy aging. Some organisms, as C. elegans, are characterized by normal long periods of starvation to respond to the onset of food shortage, and the genes responsible of this kind of environmental adaptation are the same involved in the control of lifespan. For example, the intermittent fasting (IF) extends lifespan in worms through the insulin/IGF signaling and the small GTP-ase RHEB-1. The chronic starvation has the same

effects but include the combined activity of FOXO, FOXA and AP1 (Honjoh et al., 2009; Baugh, 2013; Uno et al., 2013). The IF extend lifespan also in mice, protecting them from the neurodegenerative diseases, neurodegeneration, diabetes, hypertension, CVD and obesity (Mattson et al., 2014). In humans, the effects of IF are underway: a randomized clinical trial in pre-menopausal women showed a reduced arterial blood pressure, C-reactive protein, triglycerides, LDL, as well as fat mass, body weight and waist circumference, after fasting for two non-consecutive days per week (Harvie et al., 2011).

The isocaloric diet is a moderate-carbohydrate, moderate-fat diet that allows dieters to eat whatever they want as long as they consume the same amount of carbohydrates, proteins and fats daily, which allows quick weight loss with minimal muscle loss. Indeed, reduced calorie intake or alternative fasting with normal energy supply exerts protective effect in all tissues and organs, including brain. It was observed that in humans limiting daily food intake of an isocaloric diet in a time window to 5 to 7 hr induces more benefits compared with the standard 3 to 5 meals per day (Mattson et al., 2014). In humans it was observed that an isocaloric diet induce health benefits, probably through the activation of energy sensing pathways, such as AMPK, AKT/TOR and CREB (Mattson et al., 2014).

All these dietary intervention are focused in the reduction or the addition of some macronutrient, not considering that it is important to control the composition of the rest of the diet, as well as the age effect. In fact, in this field of study is important to take into consideration the age specific variability of nutrition effects (Wolfe et al., 2008).

2.2. Effects of nutrients.

Dietary interventions are the most studied, but scientists are investigating the role of the balance of macronutrients, because it is central and critical for the development of age-related diseases. Recent evidence, in fact, suggests that the balance of macronutrients, rather than total energy intake, plays an important role in lifespan extension (Zimmerman et al., 2003; Solon-Biet et al., 2014).

The main cause of the pathological conditions related to aging is the excess of consumption of proteins, lipids and carbohydrate and several studies have tried to figure out the association between these pathologies and diet. Currently, the “Mediterranean diet”, rich of hearty grains, olive oil, seafood, vegetables, fruits, and poor of saturated fat and meat, is considered a good diet to reduce death rates because of cancer, CVD and chronic disease (Vasto et al., 2014). In fact, it was observed that a low-carbohydrate diet reduces the body

weight and several risk factors for heart disease (Barclay et al., 2008). A lipid or glucose rich diet results in an increased IR and determines hypertension. Moreover, a high-fat diet is associated with increased mortality and increased risk of metabolic disorders (Houston, 2010).

Among nutrients, carbohydrates are fundamental organic compounds, acting as primary energy source, structural components and signalling molecules. However, a tight correlation was observed between chronic metabolic diseases and age-associated cognitive decline with a carbohydrate-rich diet, as well as the negative correlation with lifespan both in yeast and human cell cultures (Aston, 2006; Barclay et al., 2008; Seneff et al., 2011; Seetharaman et al., 2015). On the contrary, a low-carbohydrate regimen, higher in fats and with an adequate quantities of proteins, is able to improve serum factors related to the aging process (Rosedale et al., 2009). Meta-analyses of trials showed that the effects of carbohydrate restriction on weight, glycemic control and other metabolic outcomes are not preserved over time in subjects obese or with type 2 diabetes (Hashimoto et al., 2016; van Wyk et al., 2016).

A common destiny is observed for dietary lipids in relation to health and lifespan: from one side they represent the main structural compounds in biological membranes, from the other side a high-fat dietary regimen is the main susceptibility factor for metabolic diseases like type 2 diabetes and cardiovascular pathologies (Schrager et al., 2007). This is true in particular for saturated and monounsaturated fatty acids, which increase the risk of chronic diseases by triggering inflammatory responses (Mozaffarian et al., 2004), while is not for unsaturated fats, able to reduce blood pressure, blood levels of low-density lipoproteins, and increase insulin sensitivity and high-density lipoproteins (Mensink et al., 2003; Appel et al., 2005). Polyunsaturated fatty acids or PUFAs have even a favourable role in the prevention of age-related disorders and longevity promotion in model organisms and human cell cultures. Consistently, Puca and co-workers (2008) found a low level of PUFAs in erythrocyte membranes in a cohort of long-lived offspring, supporting a beneficial effect on health and longevity.

As for proteins, their effect is related to the dietary sources: a high animal-protein intake is positively associated with an increased mortality rate (75%), as well as cancer-related death risk, whereas a high plant-protein intake is negatively associated (Lin et al., 2011; Levine et al., 2014). These findings are innovative, because the old dietary guidelines promulgate high protein intake from animal sources rich in EAAs (branched chain and sulphur containing). Moreover, it is important to consider the protein turnover rates: in post-prandial moment, insulin plays a central anabolic role for muscles, while in stressful situation like inflammation, protein synthesis is increased in the liver (Attaix et al., 2005).

Several studies have been conducted to analyze the effects of both high protein intake and protein restriction (PR). In Wistar rats, CR and PR are able to decrease ROS production and DNA, protein and lipid damages: so, through the modulation of oxidative conditions PR decrease the aging rate (Ayala et al., 2007). In humans, the results of protein quantity and quality effects on molecular pathways modifications, which influence stress resistance, aging and age-related disease, are controversial. Bollwein and colleagues have observed in a population of healthy elderly in the region of Nurnberg that the distribution over the day of protein intake was significantly different among frail, pre-frail and non-frail subjects, and there was not an association between frailty and amount of protein intake (Bollwein et al., 2013).

In model organism, it was observed that there was a negative correlation between lifespan and dietary proteins (Solon-Biet et al., 2014). In humans, there is insufficient research in order to define the optimal protein intake for frail elderly. However, the current idea is to reduce protein intake in middle age and then increase it from moderate to high consumption in old age (after 65 years of age) (Levine et al., 2014). Little is known about the effects of the dietary manipulation of protein quality and quantity, in relation to the molecular pathways responsible of aging and age-related diseases; however, several studies suggested that dietary protein intake regulate the IGF-1/mTOR network, which contribute on the manipulation of these particular conditions (Efeyan et al., 2012; Fontana and Partridge, 2015). Therefore, the restriction of protein intake, rather than energy, may offer a more feasible nutritional intervention in humans, because several studies shown that they act through the same evolutionarily conserved signaling pathways. In fact, data in humans point the reduced protein intake like an important component of anti-aging and anticancer intervention (Levine, et al. 2014).

The distribution of protein throughout the day may also influence functional outcomes associated with frailty and quality of life, such as exhaustion and slow walking speed. Specifically, researchers noted that though a cohort of frail, pre-frail, and non-frail elderly individuals consumed a similar absolute and relative amount of protein each day (dietary protein consumption of all study participants exceeded the RDA), the non-frail individuals evenly distributed protein intake across their daily meals, whereas frail and pre-frail individuals skewed their protein consumption toward the noon meal (Bollwein et al, 2013).

In Japanese women, the intake of total protein was inversely associated with frailty (Kobayashi et al, 2013). Beasley and colleagues confirmed these results in a study conducted on older women (Women's Health Initiative Observational Study): they observed a lower risk of frailty after three years of high protein intakes at baseline (Beasley et al., 2013). On

the contrary, in subjects from the InCHIANTI study (a cross-sectional study of more than 800 older Italians) amount of protein intake was not associated with frailty. However, distribution of protein intake was significantly different between frail, pre-frail and non-frail participants: frail subjects showed a different and more uneven distribution of their protein intake, different by recommendations, which refers to a lower intake at breakfast, and higher intake at lunch (Bollwein et al., 2013).

Chapter 3

Nutritive and amino acid sensing.

Given the importance of nutrient homeostasis for all living organisms, and for humans in particular, it is not surprising that there are different pathways and elements, known as sensors, which detect intra and extracellular concentration of macronutrients, commonly deregulated in human disease. For all the organisms, this ability is important for the survival: in fact, in condition of food reduction they use internal resources limiting the metabolic process, while in condition of abundance the nutrient sensing pathways are activated. Studies conducted in the past years have clearly shown that the different mechanisms involved to sense nutrients with caloric value have a central role in metabolism and feeding behaviour (fundamental for survival) (Chantranupong et al., 2015). Thus, to understand the effects of specific dietary nutritional components on health and aging, it is necessary to go back to pathways regulating feeding behaviour, such as nutrient-sensing, the proper functioning of which is central to metabolic homeostasis.

3.1. Nutritive sensing pathways.

Studies of nutrient sensing focus on two area: the identification of the intracellular sensing mechanism and the characterization of elements that transport nutrient into the cell (Nicklin et al., 2009). Unicellular organisms sense both environmental and intracellular nutrient levels, because they are directly exposed to those fluctuations. In the multicellular eukaryotes, in contrast, to maintain the circulating nutrient in a specific range there are some homeostatic responses. Among the principal systems, the most important are the taste receptors, endocrine's elements, FOXO, AMPK, mTOR, PASK, FGF21, GCN2 and several membrane transporters (Efeyan et al., 2015).

Taste receptors

For some species, the first nutrient sensing event occurs at oral level by taste receptors, which are collocated on oral epithelium and sense nutrients (or macronutrients) with different mechanisms (Bachmanov and Beauchamp, 2007; Ishimaru, 2009). Interestingly, several studies have demonstrated that genes encoding sweet, bitter and umami taste receptors are also expressed in other tissues, as brain, testes, liver, kidney, pancreas, respiratory and gastrointestinal tract, with a role partially elucidated, probably related to the activation of

signaling pathways (Yamamoto and Ishimaru, 2013). In the airway, for example they have a critical role in sensing bacteria and in innate immunity regulation (Carey et al., 2016). Recent studies have actually demonstrated that bitter and sweet taste receptors, known as T2Rs and T1Rs respectively, directly participate in the immune system. Among the hypothesis for the extra oral bitter taste receptors function, one of most important is linked to their ability to bind bitter products secreted by pathogenic bacteria or fungi (Finger et al., 2003; Lee et al., 2014).

Glucose intake, storage, mobilization and breakdown are regulated at different levels by multiple mechanisms (intracellular and extracellular). The glucose taste receptor is a heterodimer composed of T1R2+T1R3 activated by millimolar concentration of the saccharides sucrose, fructose or glucose (Laffitte et al., 2014). This receptor, when activated, provokes an afferent signal to the brain, but it is also expressed in the intestinal epithelium. Here, the signal transduction does not trigger a signal to the brain, but results in the transient localization of GLUT2 transporter to the apical membrane in order to increase the absorption of glucose (Dyer et al., 2005; Mace et al., 2007).

Lipids are a large set of nutrients used for membrane biosynthesis and energy storage. The long chain unsaturated fatty acids are detected by GPR40 and GPR120, belonging to a family of G-protein coupled receptors. Through an unknown mechanism, the activation of GPR40 at the plasma membrane of pancreatic beta cells upregulates the glucose-stimulated insulin release (Itoh et al., 2003). Even the activation of GPR120 at the plasma membrane of white adipocytes promotes the cell-autonomous induction of glucose uptake by PI3K/AKT (Oh et al., 2010). This demonstrate that the increase in one nutrient anticipates the increase in another nutrient.

The AA sensing event at oral level by taste receptors (Bachmanov and Beauchamp, 2007; Yamamoto and Ishimaru, 2013). Umami taste was initially characterized as connected to monosodium glutamate. Following studies demonstrated the complex interaction with others elements: in fact, it is activated by AAs and purine nucleotides (Nelson et al., 2002). A strengthening of the activation when the two stimuli are synergic was also observed. Thus, it is reasonable to understand the existence of multiple taste receptors for umami, in particular two glutamate selective G protein-coupled receptors (mGluR1 and 4) and the heterodimer T1R1+T1R3 (Chaudhari et al., 2009). The activation of these receptors causes the release of neurotransmitters, which integrates with other events at the central nervous system level (Chaudhari and Roper, 2010).

Aging can be associated with taste disorders because of the disruption of tissue homeostasis (Heft and Robinson, 2010), like reduced number of taste buds, reduced cells per taste bud

and reduced taste bud density (Feng et al., 2014). Many studies have reported a general decline in taste sensitivity and intensity rate, although there are some controversies, because most studies show more substantial decrease in salty and bitter taste sensitivity than in sour or sweet tastes (Mojet et al., 2001; Heft and Robinson, 2010). The exact mechanism leading to these changes is still unclear; however, inflammation, gustatory nerve damage or radiation and chemotherapy may lead to taste dysfunction (Figure 4). Inflammation can induce cell death and inhibit proliferation of taste stem cells, specification and differentiation of basal cells. Both the gustatory nerve damage and the radiation or the chemotherapy can induce the degeneration of taste receptor cells, inducing apoptosis (Feng et al., 2014).

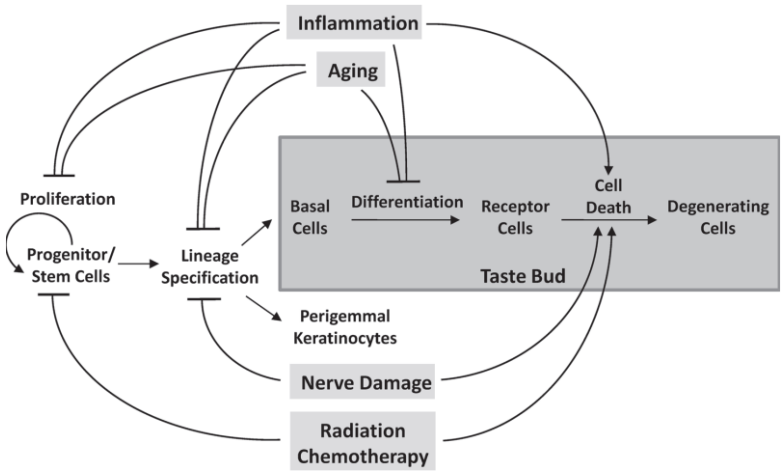

Figure 4: Effects of inflammation, aging, gustatory nerve damage, radiation, and chemotherapy on taste bud homeostasis (Feng et al., 2014).

Endocrines' elements

In pancreas, the beta cells are specialized in sensing systemic glucose levels, and are responsible for the synthesis and the secretion of insulin. In these cells, glucose is imported and phosphorylated by GLUT1 (or GLUT2) and glucokinase (GCK), and leads to an increase in ATP: ADP ratio. Therefore, GCK catalyses the first step in storage and consumption of glucose, glycogen synthesis and glycolysis and it functions as a glucose sensor (Rui, 2014). GCK has a lower affinity for his substrate, it is active only during glucose abundance and it controls systemic glucose through its effects in pancreas and liver. GCK is also expressed in the hypothalamus, which controls systemic effects such as feeding responses and insulin release, even if the role of this sensor in the brain must still be understood (Ogunnowo-Bada et al., 2014).

Adipocytes secrete hormones and adipokines, which do not necessarily reflect circulating lipid levels but exert systemic effects such as the energy expenditure or the regulation of appetite. Leptin is considered as an appetite regulator, because in the central nervous system

and in peripheral tissues coordinates food intake and metabolism. In the brain, it antagonizes the effect of appetite-stimulating neuropeptides, while in adipocytes in fasting states the leptin-decreased production promotes nutrient acquisition and stimulates appetite (Birsoy et al., 2008). Adipocytes not only synthesized leptin, but also the hormone Adiponectin, whose levels inversely correlate with lipid storage and exert systemic effects like loss of appetite, insulin sensitivity and energy expenditure (Waki and Tontonoz, 2007; Shehzad et al., 2012).

FOXO

The insulin–FOXO pathway regulates cellular growth from worms to mammals (Kenyon, 2010). FOXO integrates insulin action with the systemic nutrient and energy homeostasis through the insulin receptor substrate → phosphoinositide 3-kinase → Akt signal cascade: the phosphorylation of FOXO transcription factors by the protein kinase AKT and serum-glucocorticoid regulated kinase (SGK) is mediated by insulin and insulin growth factor 1 (IGF1) signalling. Conversely, FOXOs translocate to the nucleus when insulin and IGF1 signalling is reduced (Altintas et al., 2016).

In the liver, activation of FOXO1 induces gluconeogenesis via phosphoenolpyruvate carboxykinase (PEPCK)/glucose 6-phosphate pathway, further inhibiting mitochondrial metabolism and lipid metabolism via heme oxygenase 1/sirtuin 1/Ppargc1α pathway. In skeletal muscle, FOXO1 activation regulates the carbohydrate/lipid switch during fasting state, while its inhibition under physiological conditions promotes the maintenance of skeletal muscle mass/function and adipose differentiation. In pancreatic β-cells, conditions of low nutrition promote nuclear translocation of FOXO1, which in turn promotes the proliferation of β-cells through induction of Cyclin D1, and activates antioxidant mechanism to protect against oxidative insults. In the brain, FOXO1 controls food intake through transcriptional regulation of the orexigenic neuropeptide Y, agouti-related protein, and carboxypeptidase E. Among the four FOXO family members in mammals (FOXO1, FOXO3, FOXO4, FOXO6), the isoform FOXO3 has been linked to exceptional longevity in several independent studies (Martins et al., 2016 and references therein).

AMPK

AMPK is an important regulator of cellular metabolism because it coordinates several metabolic responses to cellular energy levels (Hardie, 2011). Specifically, AMPK is able to sense energy levels by direct binding of AMP, ADP or ATP and is activated when AMP/ATP or ADP/ATP ratios increase. In response, AMPK promotes catabolic pathways to generate more ATP while inhibiting ATP-consuming anabolic pathways. The activation of this

pathway is tightly related with the utilization of glucose: in fact, AMPK stimulates glucose uptake by muscle, promote glycolysis and mitochondrial biogenesis, and inhibits insulin secretion, gluconeogenesis and synthesis of glycogen (Hardie et al., 2012).

Lipid metabolism is another important intracellular energy source regulated by AMPK. In fact, AMPK decreases fatty acid synthesis by phosphorylating and inhibiting acetyl CoA carboxylase (ACC) enzymes, key regulators of fatty acid metabolism, but also downregulating the expression of enzymes involved in fatty acid synthesis at the transcriptional level (Mihaylova and Shaw, 2011). Evidences strongly indicated that AMPK in the hypothalamus directly acts to regulate food intake and energy metabolism by integrating hormonal and nutrient signals (Minokoshi et al, 2008).

Emerging studies also indicate that the responsiveness of AMPK signalling declines with aging (Burkewitz et al., 2014), and this can impair metabolic regulation, increase oxidative stress and inflammation, and decrease cellular autophagy, thus promoting the development of age-related metabolic diseases. By contrast, overexpression of AMPK is reported to extend lifespan of lower organisms (Salminen and Kaarniranta, 2012). In the budding yeast Saccharomyces cerevisiae, synthesis of S-adenosyl-l-methionine (AdoMet) activated the energy-sensing regulator Snf1, the orthologue of AMPK, resulting in extended lifespan (Ogawa et al., 2016). A recent work highlighted that AMPK is a key regulator of T cell function, as it promotes oxidative metabolism during metabolic stress due to changes in nutrient availability (Blagih et al., 2015). One of the downstream targets of AMPK is mTOR. The interplay between these two signalling pathways is crucial in regulating energy balance (Jia et al., 2012).

mTOR

mTOR (or mammalian target of rapamycin, now renamed as mechanistic target of rapamycin) signaling pathway is at the centre of balancing between processes which consume energy and processes which produce it (Efeyan et al., 2012; Kim et al., 2013). mTOR is a serine-threonine kinase of the superfamily of PI3KK (phosphatidylinositol-3 kinase related-kinases), which respond to stimuli as growth factors, hypoxia, nutrients, energy stress and mechanical strain. It exists in two forms: mTORC1 and mTORC2 different in their components and function (Figure 5) (Kimball and Jefferson, 2010; Laplante and Sabatini, 2012; Iadevaia et al., 2012; Ben-Sahra et al., 2013).

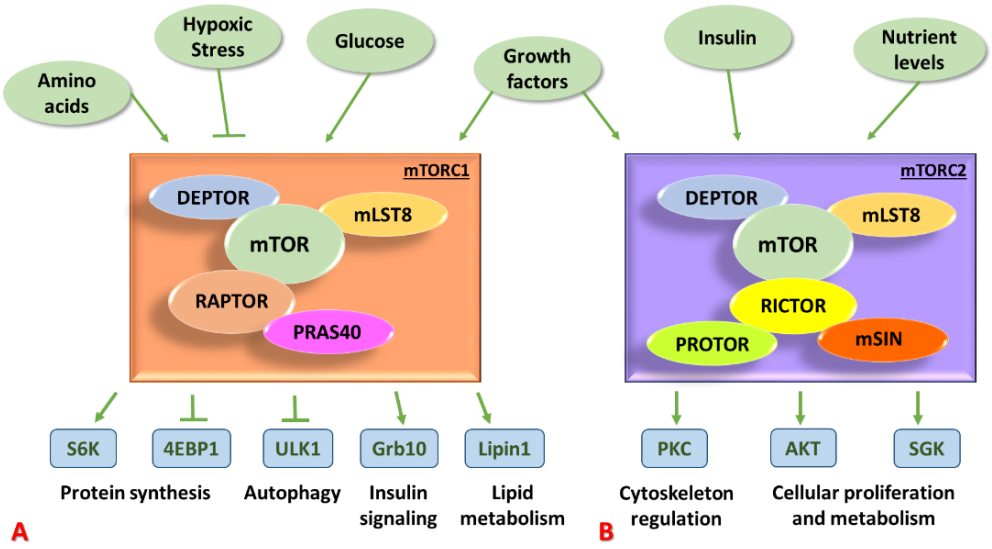

Figure 5: mTORC1 and mTORC2 pathways. mTORC1 is considered as a master nutritional sensor within cell. It senses signals from AAs, growth factors, such as insulin and insulin-like growth factors (IGF-I and IGF-II), glucose, cAMP, and oxygen. In response to such signals, mTORC1 regulates its downstream effectors to promote anabolic processes such as protein and lipid synthesis and ribosome biogenesis or to limit catabolic mechanisms such as autophagy. Regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT substrate 40kDa (PRAS40) are unique for mTORC1, while rapamycin-insensitive companion of mTOR (RICTOR), protein-observed with RICTOR (PROTOR) and mammalian stress-activated protein kinase interacting protein (mSIN1) are unique for mTORC2. mTORC2 is responsive to growth factors, such as insulin and insulin-like growth factors, via a PI3K–Akt-mediated mechanism. The regulation by insulin signalling provide a potential link between TORC1 and TORC2 activity. In addition, mTORC2 plays an important role in organizing the actin cytoskeleton and regulate cell growth and proliferation.

The activity of pro-growth signals regulates mTORC1 and the switch from catabolic to anabolic metabolism. The molecular mechanisms underlying the regulation of mTORC1 have been elucidated in the past 8 years. The energetic cost of this switch is high and, for this reason, mTORC1 is under tight regulatory control (nutrient sensing pathway and growth factors signaling) (Dibble and Manning, 2013).

mTORC1 complex is emerging as a key regulator of aging in evolutionarily divergent organisms, ranging from yeast to rodents, and it is likely that this function has been conserved also in humans. The inhibition of mTORC1-pathway components extends lifespan in model organisms and confers protection against an increasing list of age-related diseases (Stanfel et al., 2009). The complexity of the mTORC1 signalling networks presents a hurdle in determining the mechanistic details of how mTORC1 influences aging, longevity and health span. Some of the known biological processes regulated by mTORC1, including

mRNA translation and protein synthesis, autophagy, stress, mitochondrial function, and inflammation could conceivably contribute to the lifespan-extending effect of mTORC1 inhibition, possibly in a coordinated and integrated manner (Johnson et al., 2013).

On the other hand, the over-induction of mTOR pathway, leading to an increased protein production, reduced protein clearance, reduced autophagy (less clearance of protein aggregates and cellular organelles), increased protein agglomeration and inflammation can raise the risk of aging-related diseases, from AD and CVD, to atherosclerosis, neurodegenerative diseases and osteoporosis (David, 2012).

In preadipocytes, the overstimulation of the mTOR by nutrients like carbohydrates and AAs may contribute to their conversion into senescent-like preadipocytes, which secrete proinflammatory compounds (Businaro et al., 2012). The link between the activity of TORC1 and nutrient response has suggested that DR promotes longevity by reducing mTORC1 activity (Kenyon, 2010). In invertebrate organisms as well as in mammals, pharmacological or genetic disruption of mTORC1 extends lifespan under non-dietary restriction conditions (Stanfel et al., 2009; Johnson et al., 2013). Consistently, the pharmacological treatment with mTOR inhibitor rapamycine extends lifespan of 9% in male and 14% in female mice (Harrison et al., 2009).

Cellular glucose levels affect even the activity of the Rag GTP-ases, which is one of the main regulator of mTORC1 (discussed in detail above) (Efeyan et al., 2013). The mechanism involved in that is still less clear and further investigation needs to be performed, but several studies shared similar aspects downstream of glucose and AA sensing pathway, such as the involvement of the lysosomal v-ATPase (Zoncu et al., 2011; Laplante and Sabatini, 2012).

PASK and FGF21

Recent data suggests that Per-Arnt-Sim (PAS) kinase (PASK), a highly conserved serine/threonine kinase, acts as a signal integrator to somehow effectively regulate metabolic behaviour in response to energy or nutrient conditions. In particular, PAS kinase regulates lipid and glucose metabolism, mitochondrial respiration and gene expression (Cardon and Rutter, 2012; Zhang et al., 2015).

The molecular mechanism by which PASK senses cellular metabolic status and signals to regulate energy use are not yet fully determined. Consistent with its role as a metabolic sensor, studies of role of PASK in the pathogenesis of metabolic disease have identified tissue-specific metabolic phenotypes caused by PASK deletion, including impaired glucose-stimulated insulin secretion in pancreatic β-cells, altered triglyceride storage in liver, and increased metabolic rate in skeletal muscle (Hao et al., 2007). Interesting evidence indicates

that the PASK function could be critical for the normal function of AMPK and mTOR pathways (Hurtado-Carneiro et al., 2014).

An additional nutrient sensing pathways may be Fibroblast Growth Factor 21 (FGF21), which controls several metabolic functions that could be beneficial for age-related health, such as gluconeogenesis, ketogenesis, lipid metabolism, mitochondrial activity and energy expenditure. FGF21 expression is induced in response to low protein intake (Laeger et al., 2014), and in response to dietary methionine restriction (Lees et al., 2014), suggesting a role for FGF21 in linking nutrition and aging. Like AMPK and mTOR, PASK and FGF21 could be potential therapeutic targets to slow aging and to control age-related metabolic diseases in humans.

3.2. Amino acid's metabolism and sensing.

AAs are the most abundant molecules in cell. Nowadays AAs are not only considered simple proteins components (the most abundant macromolecule in cell and the second-largest component of human muscle), but also cell signaling molecules and regulators of protein phosphorylation cascade and of gene expression. Furthermore, AAs are important factors in several processes such as neurotransmitter transport and biosynthesis and in the control of protein homeostasis (Balch et al., 2008). Moreover, they have been postulated to play a critical role in growth and aging.

AAs are organic substances containing carboxyl and amine functional groups, along with a side chain (also called R group) specific to each AA (Brosnan, 2001). Among the 500 known AAs, 20 are encoded directly by the genetic code and are considered as "standard" AAs because they constitute the building blocks of protein, and two are called "non-standard" or "non-canonical" because they are encoded via variant codons. Nine of those AAs are classified as "essential" (EAA) for humans because they cannot be produced from other compounds by the human body and so must be taken in as food (Fürst and Stehle, 2004). The EAA are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and histidine. Among these, the branched chain amino acids (BCAAs; isoleucine, leucine and valine) are key regulator of protein synthesis.

Others may be "conditionally essentials" for certain ages or medical conditions. They normally can be synthesized in adequate amounts by the organism, but must be provided from the diet under particular condition in which rates of utilization are greater than rates of synthesis, such as period s of stress, aging or illness (CESSs; arginine, glycine, cysteine,

glutamine, proline, serine and tyrosine). They may differ between species: arginine, tyrosine, taurine and cysteine, for example, are considered semi-essential in children because the pathways involved to synthesize them are not fully developed (Imura and Okada, 1998). Even the amounts required depend on the age and health of the individual and for this reason is hard to make general statements about the dietary requirement for each AA. Instead, the non-essential amino acids (NEAA) are those AAs that could be synthetized de novo (Wu, 2009).

Main functions

When introduced into the human body from the diet, the 20 standard AAs can be used to synthesize biomolecules like proteins or can be oxidized to carbon dioxide and urea as a source of energy (Wu, 2009). In humans, non-protein AAs have also important roles as metabolic intermediates, such as the synthesis of GABA (gamma-amino-butyric acid neurotransmitter) or other molecules (serotonin, dopamine, epinephrine, nitric oxide, heme and some nucleotides) (Savelieva et al., 2008; Tejero et al., 2008). Table 2 shows the main functions of AAs.

AA	*Products*	*Main functions*
Alanine	Directly	Glucose-alanine cycle; gluconeogenesis; inhibition of pyruvate kinase and hepatic autophagy
β-alanine	Directly	A component of coenzyme A and pantothenic acid
	Dipeptides	Ant oxidative function
Arginine	Directly	Antioxidant; activation of mTOR signaling; activation of NAG synthase; regulation of hormone secretion and of gene expression; methylation of proteins; immune function
	NO	Signaling molecule for nutrient metabolism, angiogenesis, vascular tone, spermatogenesis, hemodynamics, mitochondrial function, etc.
	Ornithine	Mitochondrial integrity; syntheses of proline, glutamate and polyamines
Asparagine	Directly	Function of the nervous system and the immune system; regulation of gene expression; cell metabolism and physiology
	Acrylamide	Food quality; gene mutation; oxidant
Aspartate	Directly	Purine and pyrimidine synthesis; urea cycle; transamination
Cysteine	Directly	Transport of sulfur; disulphide linkage in protein
	Taurine	Osmolyte; cellular redox state; antioxidant
Glutamate	Directly	NAG synthesis; transamination; connection between urea cycle and Krebs cycle; glutamine, arginine and citrulline synthesis
	GABA	Neurotransmitter; inflammation
Glutamine	Directly	Purine, ornithine, pyrimidine, citrulline, asparagine, proline, arginine synthesis; inhibition of apoptosis; protein turnover through immune function, gene expression, mTOR signaling
Glycine	Directly	Neurotransmitter in CNS; purine and serine synthesis; calcium influx
	Heme	Production of CO; hemoproteins
Histidine	Directly	Ant oxidative dipeptides; hemoglobin structure and function; methylation
	Histamine	Allergic reaction; vasodilation; regulation of gut function
Isoleucine	Directly	Balance among BCAA; glutamine and alanine synthesis
Leucine	Directly	Regulation of protein turnover through mTOR signaling; BCAA balance; flavour enhancer
	HMB	Immune response
Lysine	Directly	Protein methylation, acetylation, ubiquitination, O-glycosylation; antiviral activity; NO synthesis regulation
Methionine	Homocysteine	Oxidant; independent CVD risk factor; inhibition of NO synthesis
	Choline	Sarcosine, phosphatidylcholine and acetylcholine synthesis
	Taurine	Anti-inflammation; antioxidant; osmoregulation; vascular, retinal, cardiac, muscular functions
Phenylalanine	Directly	BH4 and tyrosine synthesis; neurological development and function
Proline	Directly	Collagen structure and function; osmoprotectant; neurological function
	H2O2	Killing pathogens; immunity; signaling molecule

Serine	Directly	Gluconeogenesis; protein phosphorylation; one-carbon unit metabolism
Threonine	Directly	Intestinal integrity and function through mucin synthesis; glycine synthesis; immune function; protein phosphorylation and O-glycosilation
Tryptophan	Serotonin	Neurotransmitter; inflammation inhibition
	Melatonin	Antioxidant; inflammation inhibition
Tyrosine	Directly	Protein sulfation, nitrosation and phosphorylation
	Dopamine	Immune response; neurotransmitter
	Melanin	Antioxidant; inflammation inhibition
Valine	Directly	Glutamine and alanine synthesis; balance among BCAA

Table 2: Main metabolites and functions of AA (Wu, 2009).

The regulation of cell function by AA has become the focus of considerable interest. Several studies have demonstrated that increased AA availability in eukaryotic cells promotes anabolism and inhibits catabolism. When AA are scarce, those effects are reversed: the catabolic process is activated and proteins become the reservoirs of AA (proteasome-mediated degradation and autophagy) (Van Sluijters et al., 2000; Efeyan et al., 2015). The largest reservoir of peptide and free AA in the body is the skeletal muscle, which represent the 40-45% of body weight (Davis and Fiorotto, 2009), while the small intestine in humans and animals is the major site for extensive catabolism, modulating the pattern of AA in plasma and the entry of dietary AA into the portal circulation.

Several NEAA are oxidized by the enterocytes of mammalian small intestine: for example, glutamine, which is synthesized in skeletal muscle, heart, adipose tissue and placenta from BCAA, is utilized by the small intestine for the arterial circulation (Self et al., 2004). Glutamine, arginine and BCAA are metabolized in different organs and play an important role in immune function (Figure 6): from arterial blood BCAA are taken into skeletal muscle, used to synthesize alanine and glutamine and released into the circulation; the small intestine uses glutamine to synthesize citrulline (converted in the kidney into arginine). In the liver glutamate, glycine and cysteine are used for the synthesis of glutathione, and alanine for the synthesis of glucose (Li et al., 2007).

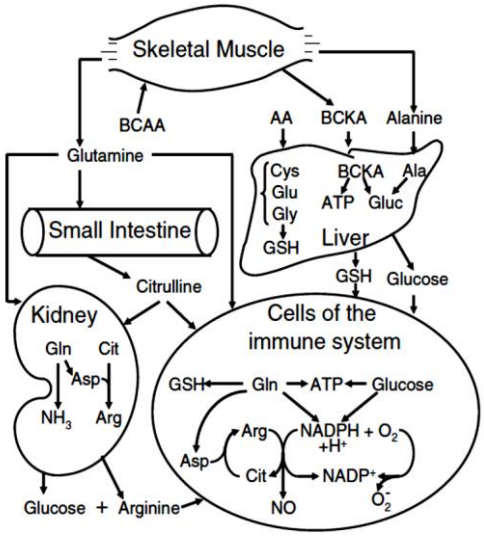

Figure 6: BCAA, glutamine and arginine metabolism (Wu, 2009).

Among BCAA, leucine is an inhibitor of protein degradation and a stimulator of protein synthesis in liver and skeletal muscle under both in vivo and in vitro experimental conditions (Suryawan et al., 2008), while isoleucine and valine have no effects on muscle protein turnover (Escobar et al., 2006). Glutamine may play an important role in mediating the BCAA anabolic effects, because valine, isoleucine and leucine are substrates of its synthesis. In rats, infusion of glutamine in skeletal muscle increases protein synthesis (Holecek et al., 2006), and the same response has been observed in chicken skeletal muscle incubated with elevated extracellular concentration of glutamine (Wu and Thompson, 1990). Even arginine increases protein synthesis in pig's muscle and in the small intestine under catabolic conditions like malnutrition or viral infection (Ban et al., 2004; Rhoads et al., 2006).

AA sensing: GCN2

GCN2 (or kinase general control nonderepresible 2) is a protein with high affinity to uncharged tRNAs and represents an example of AA sensing by the detection of a secondary molecule (Dong et al., 2000; Efeyan et al., 2015; Yuan et al., 2017). In fact, in amino-acid starved cell GCN2 is activated by all uncharged tRNAs, which lead to kinase activation and inhibitory phosphorylation of the eukaryotic initiator factor 2 alpha (eIF2α), responsible of the initiation of mRNA translation (Efeyan et al., 2015). In this way, protein synthesis is inhibited and the genes involved in AAs biosynthesis and conservation are upregulated (such as, for example, the transcriptional factor ATF4 or activating transcription factor 4), in a process known as "adaptive regulation" (Gaccioli et al., 2006). Several studies have demonstrated that in many cell types, GCN2 upregulates the *SLC38A2* expression (Gaccioli et al., 2006; Palii et al., 2006). Moreover, in several genetic, dietary and pharmacological

long-lived animal models the ATF4 stabilization by GCN2 is required for the protective effect in stress response conditions (Peng et al., 2012; Hine et al., 2015). Furthermore, it was demonstrated that GCN2 might also be involved in the mTOR signaling regulation with an unknown mechanism: the activation of GCN2 and the inhibition of mTORC1 act together to suppress the protein synthesis in condition of AA deficiency (Watanabe et al., 2007).

AA sensing: mTORC1

mTORC1 is an AA sensor: in condition of AA depletion its activity is abolished, while in AA starvation condition, mTORC1 is recruited on lysosome surface because of the action of Rag (Efeyan et al., 2012) (Figure 7).

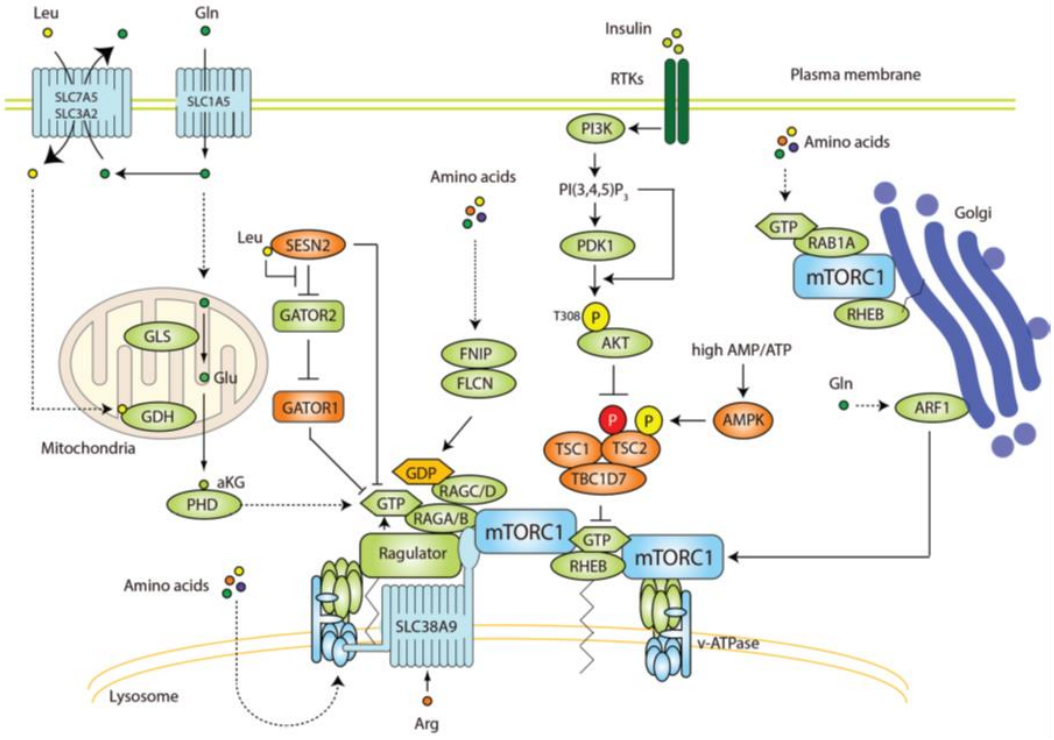

Figure 7: In mammals, essential components in an AA-sensing pathway upstream of TORC1 are four RAG (Recombination-Activating Gene) small GTPases: RAGA or RAGB (RAGA/B) forms a heterodimer with RAGC or RAGD (RAGC/D). The activation state of RAG GTPases is reflected by their guanine nucleotide binding status, and this is regulated by AAs. Under AA starvation conditions, the RAG GTPase complex is inactive and mTORC1 is dispersed in the cytoplasm. The presence of AAs promotes the formation of the active complex, in which RAG heterodimer contains GTP-bound RAGA/B and GDP- bound RAGC/D. The active RAG heterodimer binds directly to the mTORC1 component RAPTOR and redistribute mTORC1 to the surface of the lysosome where the small GTPase RHEB (endpoint of insulin and growth factors inputs via PI3K pathway) binds and activates mTORC1. AAs within the lysosome generate a signal, which is communicated to the RAGs by the activation of the v-ATPase-Ragulator complex. It has been demonstrated that the Ragulator

complex serves as a docking site for RAGs and mTORC1 on the lysosome, and functions as a guanine nucleotide exchange factor (GEF) for RAGA/B. In response to the presence of AAs, Ragulator tethers RAGs to the lysosome, which in turn relocalize mTORC1 to this organelle leading to its activation. The vacuolar H+-ATPase (v-ATPase), an ATP-driven proton pump that maintains cytosolic pH by acidifying the lysosomal lumen, interacts with RAGs and Ragulator on the lysosomal membrane and this interaction is strengthened by AA starvation and is weakened in response to AA stimulation. Thus, v-ATPase appears to act as an upstream regulator of mTORC1 in response to AAs (Shimobayashi and Hall, 2016).

It is still completely unclear whether all AAs or only a subset is monitored in the cell to activate this signaling pathway. Studies in Xenopus laevis oocytes and in mammalian cell culture have shown that intracellular AAs concentration, specifically leucine levels, are critical for mTORC1 regulation also outside the lysosome (Christie et al., 2002; Avruch et al., 2009). Sestrin-2 protein is a specific sensor for leucine: in the absence of this AA, Sestrin-2 inhibits mTORC1 by interacting with GATOR2, which represses the GAP activity of GATOR1 toward Rag-A/B. Binding of leucine disrupts the Sestrin2–GATOR2 interaction and thus allows GATOR2 to promote mTORC1 activation via inhibition of GATOR1 activity (Wolfson et al., 2016). Subsequent studies have highlighted sensitivities to other AA, such as arginine, glutamine and serine (Jewel et al., 2015; Wang et al., 2015; Fan et al., 2016). CASTOR1 regulates mTORC1 in response to arginine: under arginine deprivation, CASTOR1 forms a homodimer or a heterodimer with CASTOR2, and both complexes interact with GATOR2 to negatively regulate mTORC1 activity. Refeeding of Arginine disrupts this interaction by binding directly to CASTOR1, thereby promoting mTORC1 activation (Chantranupong et al., 2015).

The action of nutrient and growth factors on mTORC1 brings to p70 ribosomal S6 kinase (p70S6K) activation, which then leads to the phosphorylation of S6K, and stimulate eukaryotic initiation factor 4E-binding protein (4E-BP1), which results in its disassociation from eukaryotic initiation factor 4E (eIF4E) (Anthony et al., 2001; Avruch et al., 2001). This results in increased initial translation of messenger RNA (mRNA), coding for ribosomes and transcription factors, and enhanced stability (Fafournoux et al., 2000).

3.3. Amino acid transporters as sensors.

Amino acids transporters classification and function

To be absorbed, assimilated and metabolized by tissues, AAs must move through the cell membranes. However, they are not able to spread through the lipid membranes and therefore require the presence of membrane proteins called transporters that, by forming a "pore", allow them to pass. This process requires a number of steps (Figure 8): link of the AA to a specific exposed site of the transporter; conformational change in the protein that exposes the AA to the opposite side of the membrane through the pore; release of the AA and return of the transporter to the initial conformation (Taylor, 2014).

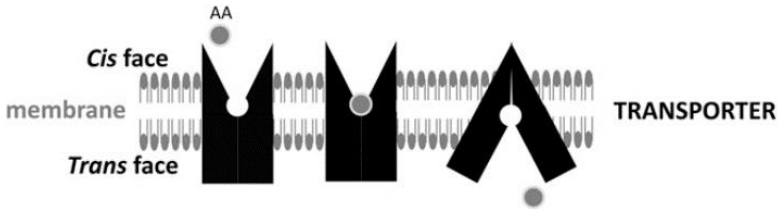

Figure 8: The transport mechanism shown in three steps (Taylor, 2014).

AAT could bind a series of structurally similar AAs, so the transport of a given AAs can be mediated by multiple transporters. In addition, the transport of a specific AAs through the membrane may depend on the integrated activity of several uniporters and antiporters that function in parallel, sometimes coupled to the ions movement such as sodium (Na +), chlorine (Cl), potassium (K +) and protons (H +). The absorption of AAs and small peptides is mediated by an active transport driven by the electrochemical gradient of Na + and H +, which is created by the Na + / K + ATPase pump located on the basolateral side of the enterocyte membrane (Poncet and Taylor, 2013). In particular, the small peptides are absorbed by means of proton and hydrolysed within epithelial cells (Gilbert et al., 2008; Nakashima et al., 2011). Neutral and anionic AAs are instead internalized thanks to a Na + symport, while the cationic / dibasic AAs are transported by antiport with other AAs. Finally, the passage of AAs from the epithelial cell to the bloodstream is counteracted by other AAs and / or facilitated effluent systems (Poncet and Taylor, 2013).

The genes of these transporters belong to a great family known since 1990 as SLC (solute carrier), according to sequence similarity and the presence of motifs family-based, which currently include 52 families and 395 transporter genes in the human genome (Hediger et al., 2013). In particular, these genes are called using a SLC code followed by a number, then a letter identifying the subfamily, and finally a number indicating the member of that

subfamily (for example, *SLC38A9*). A gene is assigned to a specific family if the coded protein has at least 20% sequence identity with other members of the family date.

Depending on its mechanism of action, transporters are divided into two groups: active and passive. The first one uses an energy-coupling mechanism, while the second one is known as facilitate transport. All these proteins were been also classified as different "systems" depending on transport mechanism, substrate specificity and regulatory properties. The first systems were defined as A and L referred to "alanine-preferring" and "leucine-preferring" (Oxender and Christensen, 1963). Then, the classification begins to differentiate between Na+-depended and –independed mechanism of transport (Bode, 2001), because several AATs belonging to System A, N and X-AG, use a secondary active transport (of Na+) to catalyse the movement, ensuring higher intracellular AA concentration (Bussolati et al., 2001). Furthermore, the principal characterization is related to the specificity for AAs and the sensitivity to inhibitors. However, at the same times, the uptake into a specific cell type of the same AA can occur through several different systems and there is a cell-specific expression of these transporters.

There are six major families of AATs: SLC1, SLC6, SLC7, SLC36, SLC38, SLC43, and there is an orphan aromatic AAT, as part of SLC16 (Table 3).

Family gene	Genes	Functional properties	Tissues localization
SLC1	*SLC1A1-7*	Belong to this family, 5 glutamate transporters involved in glutamatergic synapses and cell metabolism; and 2 neutral AATs (Kanai et al., 2013).	The expression of these proteins has been localized not only in brain but also in others tissues such as the digestive system (Avissar et al., 2001; Hashimoto et al., 2004).
SLC6	*SLC6A1-21*	This family can be divided in 4 subgroups: AATs, neurotransmitter transporters, osmolyte transporters and creatine transporters (Pramod et al., 2013).	They are expressed in neurons (*SLC6A5, SLC6A9*, in intestinal epithelial cells (neutral AATs) (Broer and Palacin, 2011).
SLC7	*SLC7A1-15*	They are divided in CAT proteins that mediate Na^+-independent transport of cationic L-AAs; and in LAT proteins that vector the transport/re-absorption of cationic AAs (Fotiadis et al., 2013).	CATs proteins are expressed in non-epithelial cells; LATs proteins are localized in placenta, brain, spleen, testes, colon (Nicklin et al., 2009; Fotiadis et al., 2013).
SLC16	*SLC16A1-14*	*SLC16A10* transports aromatic AAs and facilitates the transport of thyroid hormone T_3 (Friesema et al., 2003; Visser et al., 2011; Halestrap, 2013).	They are expressed in several tissues. *SLC16A10* is located on kidney, intestine, muscle, placenta and heart (Halestrap, 2013).
SLC36	*SLC36A1-4*	Two of these transporters (*SLC36A1-2*) function as symporters, are located in intracellular compart, cooperate to sense and reabsorb AAs (Thwaites and Anderson, 2011).	They are expressed ubiquitously but several studies have localized them in small intestine, renal proximal tubule, kidney and skeletal muscle (Chen et al., 2003; Nishimura and Naito, 2005; Heublein et al., 2010).
SLC38	*SLC38A1-11*	All these transporters carry out neutral AAs in a Na^+- dependent mechanism and are divided into system A and system N; they are also able to modulate intracellular AA pools (Franchi - Gazzola et al., 2006).	These proteins play several physiological roles in liver, brain, muscle, kidney, intestine, placenta, stem cell, immune system (Hundal and Taylor, 2009; Broer, 2014;).
SLC43	*SLC43A1-3*	This family is composed of two AATs belonging to system L and one orphan transporter. They are BCAA facilitated diffusers (Bodoy et al., 2005).	*SLC43A1* is expressed on basal membrane of trophoblast in placenta, on plasma membrane of liver and skeletal muscle, and on endoplasmic reticulum of pancreas (Fukuhara et al., 2007; Cleal et al., 2011).

Table 3: The SLC families that transports AAs (founded in HGNC) and main properties.

Their role as sensors

AATs are responsible for moving AAs in and out of a cell and between intracellular compartments. However, in addition to facilitate the delivery of AAs to the intracellular sensor molecules associated with mTORC1 and GCN pathways, new findings provide support for AATs as possible AA sensors of nutrients conditions in relation to the balancing of cellular homeostasis. For this reason, several studies were conducted in order to recognize the ones with relevant implication.

Leucine, glutamine and arginine are key stimulants of mTORC1 activation. This indicates that proteins responsible for the transport of these AAs may play an important role in the

regulation of mTORC1. Recently, it has been proposed that AA s act as “transceptors”, because they have dual transporter-receptor function (i.e., activation of an intracellular nutrient signalling cascade independent of AA transport), which enables them to sense extracellular AAs abundance (Taylor, 2014). In particular, the most studied AAT at the plasma membrane were *SLC38A2*/SNAT2 and *SLC7A5*/LAT1 (Nicklin et al., 2009; Sinclair et al., 2013), because genetic or functional inactivation of these transporters inhibits cells growth and proliferation linked to mTORC1 signaling (Pinilla et al., 2011). System ASC AA transporter 2 (*SLC1A5*/ASCT2) mostly accomplishes the transport of glutamine at the cell surface, while the influx of leucine is usually performed by system L AA transporter 1 (*SLC7A5*/LAT1). In particular, the complex *SLC7A5*/*SLC3A2* (LAT1/CD98) regulates the simultaneous transport of glutamine out of cells and the transport of leucine into cells. Glutamine deficiency up-regulates the expression of *SLC7A5* and other AATs causing a surge in the uptake of exogenous AAs, which in turn lead to mTOR activation (Chen et al., 2014).

Other cell surface L-type AATs, e.g., *SLC7A6*/LAT2, *SLC43A1*/LAT3, *SLC43A2*/LAT4 have also been linked to mTORC1 signalling (Pinilla et al., 2011). As *SLC7A5*, the other three LAT family members are responsible for the majority of cellular leucine uptake. *SLC7A6* requires the *SLC3A2* co-transporter for function whereas *SLC43A1* and *SLC43A2* are monomeric facilitative uniporters. These transporters are upregulated in multiple cancers, and are critical for control of protein translation and cell growth through the mTORC1 pathway (Wang and Holst, 2015).

There is also evidence that inhibition of these plasma membrane AATs reduces mTORC1 signalling and prevents growth and proliferation of mammalian cells in culture (Nicklin et al., 2009; Pinilla et al., 2011; Sinclair et al., 2013).

*SLC38A2/*SNAT2 is localized at the plasma membrane and transports small neutral AAs such as serine, alanine and glutamine, thus having overlapping substrate specificity with *SLC1A5*. In response to extracellular AA limitation, the expression of *SLC38A2* is up-regulated by a mechanism dependent on the activating transcription factor 4 (ATF4), while the activity is increased by its enhanced stabilization (Hyde et al., 2007). Additionally, it may activate mTORC1 through a signalling mechanism independent of increases in intracellular AA concentration, thus acting as a putative AA transceptor (Pinilla et al., 2011).

Recent findings indicate that AATs on lysosomal membranes also play a role in AAs sensing. Several reported cytosolic AAs sensors bind directly AAs as leucine and promote through various reactions the recruitment of mTORC1 on lysosome membrane (Han et al., 2012). A recent study has demonstrated an association between mTORC1 related proteins

recruitment and AAs accumulation on lysosome, underlying the importance of the endosomal transporters (Yu et al., 2010). In particular, leucine flux into the lysosome is mediated by the heterodimer *SLC7A5-SLC3A2*, where it is recruited through association with the lysosomal-associated transmembrane protein 4b (LAPTM4b), leading to activation of mTORC1 (Milkereit et al., 2015).

Two others AATs, the H+-coupled AAT *SLC36A1*/PAT1 and the *SLC38A9*/SNAT9, have been reported to function as transceptors on the lysosomal membrane. In vivo study conducted on flies has identified a unique over expressed AAT belonging to SLC36 family (PAT) as responsible of growth and physiological activity. Other studies demonstrated that PAT use the transport of AAs for activating mTORC1 partway, acting as a "transceptor". Therefore, we can better understand that this protein is able to sense all AAs, not just the one that it transports (Heublein et al., 2010; Ögmundsdóttir et al., 2012). In particular, *SLC36A1* has a double collocation (endosomal and plasma membrane) and several studies have demonstrated that PAT1 is needed for AA-induced activation of mTORC1 pathway, but exert a negative effect when overexpressed (Heublein et al., 2010; Zoncu et al., 2011). Recently, a member of the PAT/SLC36 family, *SLC36A4*/PAT4, has been shown to be mainly localised on the trans-Golgi network, where interacts with the small GTPase Rab1A to activate mTORC1, likely by sensing the intracellular levels of glutamine and serine (Matsui and Fukuda, 2013; Fan et al., 2016). However, several studies demonstrated that not only PAT, but also different AATs have important roles on mTORC1 and GCN pathways (upstream and downstream) (Kriel et al., 2011; Kim et al., 2013). In particular, in AAs abundance has been proposed a model of lysosomal "nutrisome" as a sensor of upstream concentration composed by v-ATPase (vacuolar H+-ATPase) and PAT1 (Zoncu et al., 2011).

Recently, even *SLC38A9* coding for SNAT9 that transports mainly arginine, has been characterized as a component of the lysosomal AA-sensing machinery, through proteomic analysis (Chapel et al., 2013), able to contribute in growth regulatory pathways as a transceptor-type of SLC (Rebsamen et al., 2015), through direct interaction with mTORC1 (Wang et al., 2015). Gain of function of this transporter makes cells resistant to AA withdrawal, while loss of *SLC38A9* expression impairs AA-induced mTORC1 activation, suggesting that SNAT9 is an arginine sensor (Rebsamen et al., 2015). Mass spectrometric analyses find the isoform 1 (*SLC38A9.1*), with sequence similarity to the SLC38 class of sodium-coupled AATs, different to the isoform 2 (Sundberg et al., 2008). In particular, it was observed that only the *SLC38A9.1* is able to interact with mTORC1 complex (Wang et al., 2015). Recent analysis in rats has identified *SLC38A7*/SNAT7 as AATs on lysosomes (Chapel et al., 2013), as SNAT9.

Chapter 4

Influence of protein balance on age-related traits: frailty and sarcopenia.

As society ages, the incidence of physical disability substantially increased among adults. In fact, approximately 30% of the population 55 years and older are facing with moderate or severe physical disabilities. This physical disability decreases quality of life, increases the risk of institutionalization and hospitalization and leads to premature death. In addition, the higher age-related prevalence of physical disability will increase the demand on our health care system. Nutrition is one of the most important contributing factors to the loss of independence. Furthermore, the physiological changes that characterize the aging process may have an adverse effect on dietary composition at old age (Dato et al., 2013). Malnutrition plays a key role in the pathogenesis of the most important age-related disabling phenotypes: frailty and sarcopenia (Cruz-Jentoft et al., 2017; Lorenzo-Lopez et al., 2017). Older adults, in particular, experience energy lower intake due to a reduction in appetite or anorexia of aging (Morley, 2013; Landi et al., 2016), and usually they eat less protein than younger adults (Volpi et al., 2013), thus requiring protein supply. Nutrient balance, in particular protein intake, has direct effects on skeletal muscle because of its role as a reservoir of AAs (that can support energy production and protein synthesis), as well serving as a regulator of inter-organ crosstalk (Argilés et al., 2016).

Frailty and sarcopenia

Frailty is a biological syndrome of decreased reserve and resistance to stressors. The term was first introduced in medical literature in 1970 and is receiving increasing focus in research and clinical practice due to its ability to predict poor outcomes in older people. In 1994, Rockwood and co-workers developed the accumulated deficit model of frailty (Rockwood et al., 1994), which considers the interaction between components that help an elderly person to survive (biomedical factors such as health, illness and disability) and components that hurt their ability to live independently (psychosocial factors such as resources available and dependence on others). This theory has allowed the developing of tools to assess the degree of frailty, indicated as frailty indexes, valid in both individuals and populations (Searle et al., 2008). These indexes included more than 70 potential deficits, such as psychological symptoms, ability in daily life activities and aspects of general health and disease. Several studies provided evidences in support of the accumulated deficit model, like that from

Mitnitski who found an association between time of death and increasing frailty index score, highlighting the role of the concept of frailty above that of age to predict adverse outcomes in elderly (Mitnitski et al., 2001; Milte and Crotty, 2014).

In 2001, Fried and co-workers provided a standardized definition of frailty and this opened new opportunities in the study of the biological basis of human aging. According to this definition, frailty should be considered as a cycle syndrome that includes the reduction of resting metabolic rate, the reduction of total energy expenditure, a chronic under nutrition and sarcopenia (Figure 9).

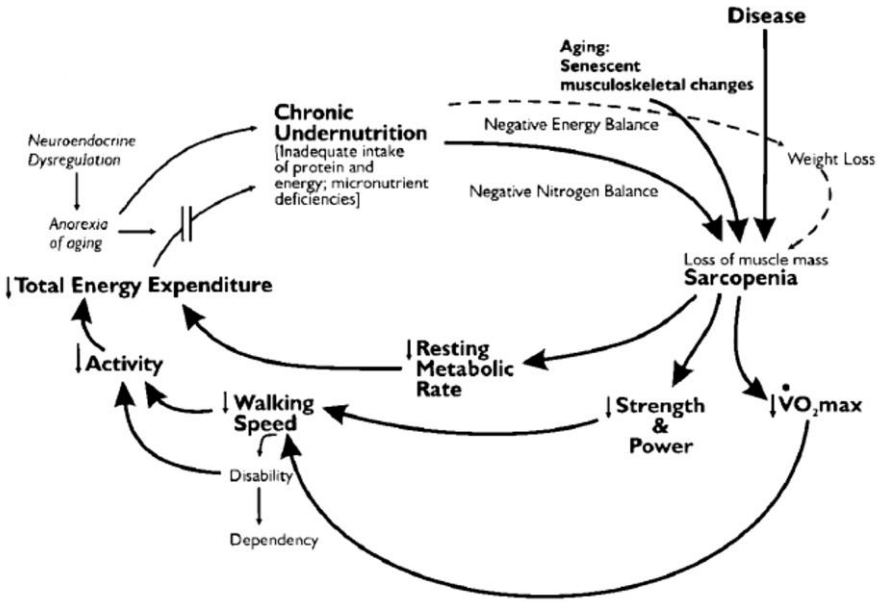

Figure 9: Cycle of frailty (Fried et al., 2001).

The innovative point of Fried's study is the definition of some criteria, listed in Table 4, relatively easy and inexpensive to apply, which offer a basis for screening frailty in older adults at risk, and for establishing clinical risk of adverse outcomes. These criteria also provide a phenotype applicable to future research on aetiology and interventions to prevent or delay the progression of frailty. Following these criteria, a frailty status is present when you can find at least three of five risk factors, among weight loss, exhaustion, decrease of physical activity, increased time of walking and decreased grip strength, but psychological, cognitive and social factors can also contribute to the development of this syndrome (Pel-Littel et al., 2009).

Weight loss At follow-up, weight loss was calculated as: (Weight in previous year – current measured weight) / (weight in previous year) = K. If K ≥ 0.05 and the subject does not report that he/she was trying to lose weight (i.e., unintentional weight loss of at least 5% of previous year's body weight), then frail for weight loss = Yes.	"In the last year, have you lost more than 10 pounds unintentionally (not due to dieting or exercise)?" If yes, then frail for weight loss criterion.	
Exhaustion Using the CES–D Depression Scale, the following two statements are read: (a) I felt that everything I did was an effort; (b) I could not get going.	The question is: "How often in the last week did you feel this way?" 0 = rarely or none of the time (< 1 day), 1 = some or a little of the time (1–2 days), 2 = a moderate amount of the time (3–4 days), 3 = most of the time. Subjects answering "2" or "3" to this question are categorized as frail by the exhaustion criterion.	
Physical Activity Kcals per week expended are calculated using standardized algorithm. This variable is stratified by gender.	Based on the short version of the Minnesota Leisure Time Activity questionnaire, asking about walking, chores (moderately strenuous), mowing the lawn, raking, gardening, hiking, jogging, biking, exercise cycling, dancing, aerobics, bowling, golf, singles tennis, doubles tennis, racquetball, calisthenics, swimming. Men: Kcal of physical activity per week < 383 are frail. Women: Kcal of physical activity per week < 270 are frail.	
Walk Time Stratified by gender and height.	Men Height ≤ 173 Height > 173	Cut-off for time to walk 15 feet: ≥ 7 sec ≥ 6 sec
	Women Height ≤ 159 Height > 159	Cut-off for time to walk 15 feet: ≥ 7 sec ≥ 6 sec
Grip Strength Stratified by gender and body mass index (BMI) quartiles.	Men BMI ≤ 24 BMI 24.1 – 26 BMI 26.1 – 28 BMI > 28	Cut-off for frailty: ≤ 29 ≤ 30 ≤ 30 ≤ 32

	Women	Cut-off for frailty:
	BMI ≤ 23	≤ 17
	BMI 23.1 – 26	≤ 17.3
	BMI 26.1 – 29	≤ 18
	BMI > 29	≤ 21

Table 4: Criteria used to define frailty by Fried and co-workers (Fried et al., 2001).

An interesting aspect emerged from the study conducted by Fried is that frailty is associated with several chronic diseases or comorbidities and the possible mechanisms by which individuals became frail may be two: as a result of physiological changes (not disease-based), and because of severe diseases (in this case they could potentially initiate frailty via any point on the hypothesized cycle). However, frailty not only is associated, but it could be an outcome of some age-related diseases. In fact, several studies show that comorbidities like rheumatoid arthritis, hypertension, congestive heart failure, myocardial infarction, diabetes and peripheral vascular diseases increase the risk for frailty (Newman et al., 2001; Klein et al., 2005). Fried and co-workers used the definition of "primary" and "secondary" frailty to refer to frailty in absence or presence of chronic disease (Fried and Walston, 2003). Thus, it is possible to hypothesize that frailty leads these diseases in a subclinical manner, such that it manifests itself concomitantly with the illness itself (Fulop et al., 2010).

The causes of frailty are complex and they must be based on the interplay of genetic, biological, physical, environmental, social and psychological factors (Walston et al., 2006; Rockwood and Mitnitski, 2007a,b). Only few altered physiological pathways can be related to frailty and could be considered as causing factors for the other age-related disease. One of the most important seems to be the altered inflammatory response, which leads to the development of clinically significant pathologies and to a number of symptoms such as unintentional weight loss, low muscle strength and exhaustion. These conditions are accompanied by an increase of pro-inflammatory markers, especially IL-6 (Fulop et al., 2010), one of the most studied in relation to frailty, together with its surrogate C-reactive protein (CRP). Several large studies have also demonstrated that some lipid parameters could also been associated with frailty syndrome: in particular, decreased total cholesterol and HDL-C were the most strongly associated (Schalk et al., 2004; Landi et al., 2008).

Physical decline and disability remains one of the key predictive features in the development of frailty phenotype (de Vries et al., 2011; Morley et al., 2013). This physical impairment, determined by the loss of muscle mass and strength, and associated to atrophy of the type II fiber, necrosis and reduction of cross-bridging elements between fibers, represents the first preliminary stage of a process potentially driving the individual toward more severe

disability (Lang et al., 2010; Cesari et al., 2014). Such complex muscle alterations with age is known as "sarcopenia" (Rosenberg, 1997). In 2010, the European Working Group on Sarcopenia (EWGSOP) defined it as a multi-system syndrome and developed a clinical definition and consensus diagnostic criteria for sarcopenia, based on gait speed, grip strength and muscle mass measurement (Cruz-Jentoft et al., 2010). The degenerative loss of skeletal muscle happens at a 3−8 percent rate each decade after 30 years old and accelerates with advancing age. The onset of physiologic and behavioural changes that precede sarcopenia can occur relatively early, during the fourth to fifth decade of life (Breen and Phillips, 2011). Though the epidemiology of sarcopenia is highly dependent on the definition applied, sarcopenia occurs in similar proportions in men and women, with a higher prevalence among individuals aged 70 and over (Beasley et al., 2013 and references therein). In both men and women, a decline in lean body mass seems to become detectable after the age of 45 years (Morley et al., 2013). Sarcopenia is also associated with major co-morbidity such as osteoporosis, obesity, insulin resistance, type 2 diabetes, malnutrition, cancer, chronic pulmonary and kidneys disease, immune disorders, sepsis, muscular dystrophies, burns and forced immobilization (Gielen et al., 2012; Santilli et al., 2014).

The precise etiology of sarcopenia has yet to be elucidated, although several evidence suggested a number of putative mechanisms likely involved in the onset of sarcopenia. As showed in Figure 10, inactivity can lead to loss of the skeletal muscle mass and atrophy. Furthermore, mitochondrial dysfunction, insulin resistance and inflammation, and decreased level of anabolic hormones are regarded as common linking factors associated with this phenotype. Finally, impaired skeletal muscle maintenance can depend on the unbalance between the proteolytic processes (muscle protein breakdown, MPB) and the protein synthesis (muscle protein synthesis, MPS) (Fry and Rasmussen, 2011).

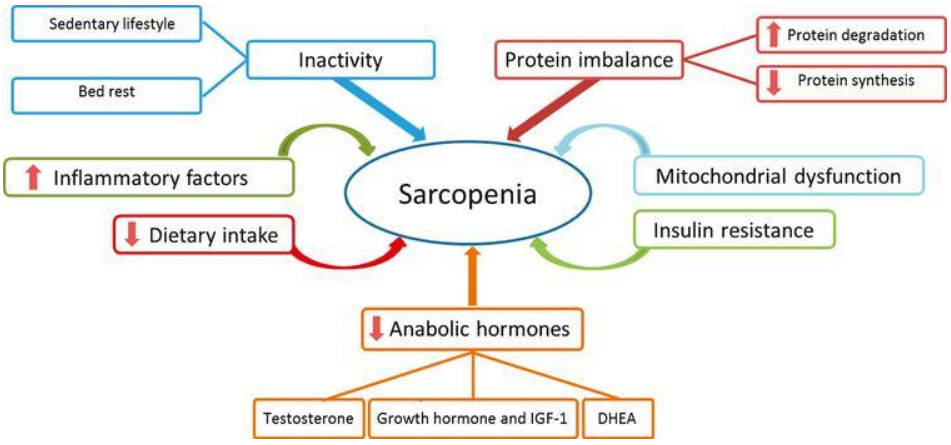

Figure 10: Main mechanisms involved in sarcopenia (Farshidfar et al., 2015).

Studies focused on assessing rates of MPS and MPB in response to anabolic stimuli reported lower rates of MPS in the elderly, highlighting potential age-related differences in such a response (Figure 11).

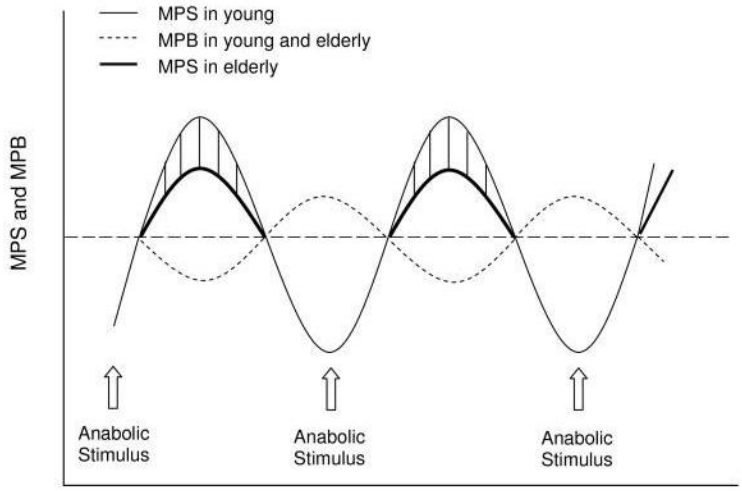

Figure 11: Skeletal muscle metabolism in response to anabolic stimuli (AA ingestion and/or physical activity) in young and elderly (Breen and Phillips, 2011).

A possible explanation is that, with aging, muscle cells loss the ability to respond to the anabolic stimulus, thus manifesting the so-called "anabolic resistance", with an increase of the "anabolic threshold" required to maximize anabolic pathways (Dardevet et al., 2012).
However, other factors can affect muscle protein turnover (i.e. physical activity). In fact, during periods of muscle disuse/unloading due to illness or hospitalization that occur with increasing frequency in the elderly, the rate of muscle loss is exacerbated (Fry and Rasmussen, 2011; Breen and Phillips, 2011).
Due to its multifactorial nature, due to environmental and behavioural, and genetic factors, a large inter-individual variability in sarcopenia has been observed (Katzmarzyk, 2010). While the effects of environmental factors have been broadly investigated, only recently studies have begun to investigate the specific genetic influences in order to explain the inter-individual trait variability. In particular, a number of candidate genes have been associated with muscle phenotypes in the elderly (Roth, 2012; Garatachea and Lucía, 2013). Dato and co-workers (2012) on a Danish-twin study showed the presence of genetic influence on the variance of frailty: in men, in particular, this status is more linked to the genetic background (43%) than in women, where it is greater the influence of the environmental component. However, the collected data shows that most of the eligibility observed for the frailty indicators is related to the inheritance of muscle strength: handgrip in fact has a 52% inheritance in Danish twins in both sexes (Dato et al., 2012). This observation has been

confirmed by other studies, which established a heritability of muscle-related traits between 30 and 85% for muscle strength and between 45 and 90% for muscle mass (Roth, 2012). In addition, several studies showed that the influence of environment on this trait is reduced with age and the genetic influence became the most important (Passarino et al., 2006).

Protein/amino acids balance in physical disability

A hot topic in gerontology is to understand the specific nutritional factors and cellular mechanisms that may facilitate the stimulation of MPS in older adults. An overarching hypothesis generated during the Protein Summit 2.0 was that consuming an adequate amount of high-quality protein at each meal, in combination with physical activity, may delay the onset of sarcopenia, slow its progression, reduce the magnitude of its functional consequences, or all of these (Paddon-Jones et al., 2008).

As components of proteins, the AA availability controls, mainly through the mTORC1 signaling pathway, the muscle protein metabolism improving the rate of muscle protein synthesis (Dickinson et al., 2011). The anabolic action of AAs on muscle proteins is due mainly to the EAAs, which appear to be the most efficient nutrient font in the stimulation of muscle protein synthesis in older and in younger subjects. Besides intake of EAAs, there are also lots of evidence suggesting that resistant exercise can stimulate growing of muscle mass through direct stimulation of muscle protein synthesis, which cause contractile protein accumulation and hypertrophy of individual muscle fibers. While there is ample evidence supporting that resistance-exercise training is a valuable intervention to induce muscle hypertrophy in young people, the response of muscle to EAAs ingestion combined with resistance exercises is diminished in elderly compared to young people (Fry et al., 2011; Brook et al., 2016).

Among EAAs assumed to suppress age-related muscle loss by regulating MPS and to prevent sarcopenia, the circulating BCAA levels have been positively correlated to protein intake under chronic feeding condition (Fujita and Volpi, 2006). In fact, BCAAs, and in particular leucine, increase fat leptin secretion, decrease food intake and body weight via mTORC1 signaling, and improve muscle glucose uptake and whole body glucose metabolism. Leucine in particular, is both a potent activator of protein synthesis and an inhibitor of proteolysis, forcing muscle fibers to favor the anabolic phenotype (Valerio et al., 2011).

Furthermore, because of the physiological occurrence of muscle loss with aging, the stores of further EAAs is reduced in skeletal muscle thus contributing to sarcopenia; for instance, in stress conditions occurring with aging, a low concentration of glutamine in plasma is

considered a hallmark of catabolic states (Meynial-Denis, 2016). However, in supplementation, concentrations of BCAAs should be carefully controlled, considered that while reduced levels of BCAA were associated in mice with life-span extension (Solon-biet et al., 2014), human epidemiological studies show that elevated BCAA levels are associated with heart disease, obesity and diabetes, likely through an over-activation of mitochondrial biogenesis and nutrient signaling pathways (Solon-biet et al., 2015). As a further example, the supplementation with the BCAA leucine, key regulator of translation initiation in MPS, appears to be the most effective treatment in numerous muscle wasting conditions associated with aging, cancer and immobilization (Churchward-Venne et al., 2014). At the same time, a prolonged leucine supplementation study in healthy elderly did not show any enhancement in muscle mass, suggesting that a high quality leucine rich proteins maximize MPS, while free leucine addition does not appear effective (Chuchward-Venne et al., 2012). Moreover, in elderly people, evidences demonstrated that dietary supplementation with proteins or AAs is inefficient in limiting the atrophy processes in absence of resistance-type exercise training (Evans et al., 2013). For example, the post-exercise leucine assumption can extend the skeletal muscle sensitivity and anabolic response to AAs, making this a potential therapeutic program (Dickinson et al., 2014). Nevertheless, the combination of free leucine supplementation with RE in elderly shows moderate and positive changes in muscle strength and in certain component of functional status parameters (Trabal et al., 2015).

The transport of AA is critical for their supply to all tissues and the skeletal muscle homeostasis as they may have a key role in such process as AA sensors (Dickinson and Rasmussen, 2013). As previously discussed, this is likely linked to the ability of AATs to act as both transporter and receptor (transceptor) (Taylor, 2014; Goberdhan et al., 2016) able to transduce a signal reflecting AA availability and leading to activation of mTORC1 (Shimobayashi and Hall, 2016).

The first study to characterize AAT expression in human skeletal muscle following AA ingestion was performed in 2010 by Drummond et al. (Drummond et al., 2010). They showed that EAA ingestion increased blood leucine concentration, delivery of leucine to muscle, transport of leucine from blood into muscle, intracellular muscle leucine concentration, ribosomal protein S6 (Ser240/244) phosphorylation, and muscle protein synthesis. This was followed by increased mRNA expression of *SLC7A1*/LAT1, *SLC3A2*/CD98, *SLC38A2*/SNAT2, *SLC36A1*/PAT1, and increases in LAT1 and SNAT2 protein expression. These increases are likely occurred downstream of mTORC1 signaling, suggesting a cellular adaptation mechanism to improve AA transport and, thus, mTORC1 signaling, during prolonged AA sufficiency or in response to a future protein anabolic

stimulus. The expression of the above transporters in human skeletal muscle has also been investigated in response to resistance exercise, demonstrating that resistance exercise increases the expression of select AATs in the skeletal muscle of both young and older individuals (Drummond et al., 2011). Consistently, it was observed that bed rest in older adults decrease the AA induced MPS through the downregulation of mTORC1 and AATs content (Drummond et al., 2012). Subsequently, Dickinson and co-workers (2013) showed that aging differentially affected the expression of LAT1 and SNAT2 in the skeletal muscle when EAAs were ingested after exercise, suggesting that aging may influence the function of specific AATs, and possibly age-related phenotypes, such as sarcopenia. All together, the above studies support the hypothesis that the activity of AATs provides an important age-related link between AA availability and regulation of muscle protein metabolism.

Chapter 5

Aim of the study.

Based on the recent important findings that showed the age-related effects of AAs availability and physical performance on the genes expression levels of specific AATs (stated in the introduction section), the goal of this thesis was to gain a better understanding of the role of AATs on the risk to develop physical disability.

To this aim, we screened 58 genetic variants occurring in ten selected genes encoding for AATs in a population of elderly subjects aged 50-89 years, analysing them in combination with parameters of physical performance. The parameters analysed were the HG, a very reliable marker of the functional status, as well as one of the most effective predictors of disability and mortality in the elderly, the ADL, which is essential to a satisfactory health-related quality of life and WS, also a strong predictor of adverse health outcomes. Taking advantage of the longitudinal study design of the analysed cohort, for all the variants analysed we investigated the association with survival at 120 months. In addition, to investigate whether variants in gene encoding AATs also concur to determine the chance to reach very advanced age, we performed a cross-sectional study by including subjects aged 90 years and older.

Chapter 6

Physical decline and survival in the elderly are affected by the genetic variability of amino acid transporter genes

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Abstract

Amino acid (AA) availability is a rate-limiting factor in the regulation of muscle protein metabolism, and hence a risk factor for age-related decline in muscle performance. AA transporters are emerging as sensors of AA availability and activators of mTORC1 signalling, acting as transceptors. Here, we evaluated the association of 58 single nucleotide polymorphisms (SNPs) in 10 selected AA transporter genes with parameters of physical performance (Hand Grip, Activity of Daily Living, Walking time). By analysing a sample of 458 subjects aged 50-89 years, we found significant associations with *SLC7A5*/LAT1, *SLC7A8*/LAT2, *SLC36A1*/PAT1, *SLC38A2*/SNAT2, *SLC3A2*/CD98, *SLC38A7*/SNAT7 genes. Further investigation of the SNPs in a cross-sectional study including 271 subjects aged 90-107 years revealed associations of *SLC3A2*/CD98, *SLC38A2*/SNAT2, *SLC38A3*/SNAT3, *SLC38A9*/SNAT9 variability with longevity. Finally, a longitudinal study examining the survival rate over 10 years showed age-dependent complexity due to possible pleiotropic effects on different phenotypes for SNPs in *SLC36A1*/PAT1, and trade-off dynamics for a SNP in *SLC38A9*/SNAT9, conferring a survival advantage before 90 years of age and disadvantage later.

On the whole, our findings support the hypothesis that AA transporters may impact on the age-related physical decline and survival at old age in a complex way, likely through a mechanism involving mTORC1 signalling.

Introduction

One of the most dramatic modifications associated with human aging is the progressive decline in skeletal muscle mass and function, known as sarcopenia, a downward spiral that causes physical function impairment, leading to a loss of functional independence and to an increased incidence of adverse health outcomes [1, 2]. In fact, the high prevalence of this condition due to the prolonged life expectancy led in the recent decades to increased needs for health care services and resources to support older people [3]. The rate of skeletal muscle loss is estimated at 8% per decade from the 4th until 7th decade, with about 15% lost each decade after 70 years of age and loss of strength is estimated to be even longer [4].

The cause of sarcopenia is widely regarded as multifactorial, with chronic diseases, hormonal and inflammatory changes, mitochondrial dysfunction, physical inactivity, and malnutrition, being the principal risk factors [5]. In addition to these causes, loss of muscle has been related to the imbalance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), associated with alterations in muscle anabolic responses to nutritional stimuli and physical activity, the so-called "anabolic resistance" according to which in the elderly process the "anabolic threshold" required to maximize anabolic pathways is increased [6, 7].

Amino acids (AAs) availability is among the most important anabolic signals for MPS [8], and essential amino acids (EAAs), leucine in particular, has been shown to be critical in the regulation of skeletal muscle protein synthesis and degradation in the elderly [9]. There is growing evidence that AA transport into muscle cells may be a rate-limiting step in the process of AA induced stimulation of skeletal muscle protein metabolism, and that AA transporters may have a key role in such process as AA sensors [10]. This is likely linked to the ability of AA transporters to act as both transporter and receptor (transceptor) [11, 12] able to transduce a signal reflecting AA availability and leading to activation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1), the major nutrient-sensitive signalling pathway [13]. Thus, AA transporters may be an important link in the ability for AAs to stimulate MPS. This has been supported by studies in human skeletal muscle showing that the expression of AA transporters is highly dynamic and responsive to different anabolic stimuli [14]. In particular, in 2010, Drummond and colleagues [15] characterized the expression of selected AA transporters in the human skeletal muscle following AA ingestion in a group of healthy young individuals. They found increased mRNA expression of L-type AA transporter *SLC7A5* (LAT1), *SLC3A2* (CD98), sodium-coupled neutral AA transporter *SLC38A2* (SNAT2), and proton-coupled amino acid transporter *SLC36A1* (PAT1), which are transporters thought to have key roles in mTORC1

signalling regulation and muscle protein synthesis and muscle growth. These authors argue that, changes in the expression levels of AA transporters, possibly mediated by mTORC1 activity, could serve as an adaptive response for improving AA intracellular delivery and for regulating the rate of muscle protein synthesis in response to anabolic stimuli or during periods of decreased muscle protein synthesis [15]. Consistently with this hypothesis, the same authors showed that an up-regulation of the above transporters occurred after resistance exercise in both young and older adults, likely regulated in an age-dependent manner [16], as well as in older persons in conditions of short-term bed rest [17]. Soon after that, Dickinson and co-workers [14] reported that aging differentially affected the expression of *SLC7A5* and *SLC38A2* in the skeletal muscle when EAAs were ingested after exercise, suggesting that aging may influence the function of specific AA transporters, and possibly age-related phenotypes, such as sarcopenia [14, 18].

Based on these evidences, to shed a light on the impact of the above AA transporters on the age-related loss of muscle strength and physical performance, we screened genetic variants occurring in their genes in a population of elderly subjects, analysing them in combination with parameters of physical status. Moreover, beside these four genes [*SLC7A5* (LAT1), *SLC3A2* (CD98), *SLC38A2* (SNAT2) and *SLC36A1* (PAT1)], we also included variants located in *SLC7A8* (LAT2) and *SLC43A1* (LAT3) as leucine transporters like LAT1, *SLC1A5* (ASCT2) as a glutamine transporter like SNAT2, *SLC38A3* (SNAT3) and *SLC38A7* (SNAT7), preferentially transporting glutamine and arginine, *SLC38A9* (SNAT9), as an arginine transporter [19, 20].

Taking advantage of the longitudinal study design of the analysed cohort, for all the variants analysed we investigated their association with survival at 120 months. In addition, to investigate whether variants in above gene also concur to determine the chance to reach very advanced age, we performed a cross-sectional study by including subjects aged 90 years and older.

Results

Demographic, clinical, and anthropometric characteristics of the analysed sample are presented in Table 1. Among the selected SNPs, 10 did not pass the QC phase. In particular, four SNPs were excluded due to a MAF (Minor Allele Frequency) lower than ten percent per locus (rs17112008, rs7968173, rs1175, rs7735053), while six were excluded because it showed a significant deviation from HWE in control subjects (rs11749532, rs7736177, rs2897968, rs17794251, rs7193392, rs8058969).

Association with muscle-related phenotypes

Complete results of the association tests in the 50-89 years old cohort are reported as Supplementary Figure 1 (Figure S1). In Table 2 are reported the SNP set showing association with at least one trait under study at nominal significance ($p^{Model} < 0.05$).

Association with HG performance was found for LAT genes *SLC7A5* rs4329925 T/C (β = -1.98, p^{Dom} = 0.027), and for *SLC7A8* rs999165 T/A (β = -3.091, p^{Dom} = 0.0013) and rs12588118 C/G (β = -6.41, p^{Rec} = 0.002). For these SNPs, the minor allele was associated with a decreased HG performance as indicated by the corresponding regression coefficients. *SLC7A5* and *SLC7A8* variability was also associated with ADL performance. Regarding *SLC7A5*, we found association with rs731710 A/G, while for *SLC7A8* the variant most significantly associated was rs3783436 T/C. For both SNPs, the minor allele was conferring an increased ability to perform physical activities with an OR of 0.47 (CI 0.27-0.81, p^{Add} = 0.005) and 0.40 (CI 0.20-0.82, p^{Add} = 0.007) per risk allele, respectively. A positive effect on ADL performance was also observed for two SNPs in *SLC36A1*, rs357618 A/G and rs357629 A/G. For both the OR was 0.11 (CI 0.01-0.96; p^{Rec} = 0.013). Analysis of LD indicated these two SNPs to be strongly correlated with each other (r^2 = 0.97). Therefore, these associations are not independent.

Regarding *SNAT* genes, a strong association with ADL scores was found for *SLC38A2* rs1873793 T/C, with CC homozygous subjects having a higher risk to become disable (OR 3.06, CI1.36-6.85; p^{Rec} =0.007).

In the case of WT performance, two SNPs in *SLC3A2*, rs12804553 G/T and rs4726 C/T, showed an opposite effect: subjects with two copies of the less frequent allele were associated, respectively, with more (β = +2.48; p^{Rec} = 0.029) and less (β = -1.40; p^{Dom} = 0.034) walking time. Another positive significant association was found for *SLC38A7* rs9806843 (β = +1.70; p^{Dom} = 0.008).

Association with longevity

To investigate whether variants in gene encoding AA transporters also concur to determine the chance to survive and/or to reach very advanced age we applied both a cross-sectional study, by including 271 more subjects aged 90 years and older, and a longitudinal assessment of survival by genotype, in the whole analysed sample.

Results of case-control analysis are shown in Table 3. Among the SNPs showing association with at least one muscle-related phenotype, in accordance with the negative effect of the minor C allele of rs1873793 (*SLC38A2*) on ADL performance, subjects carrying this allele were also significantly less frequent in the older population compared to the younger one (OR=0.70, CI 0.5-0.95; p=0.035). Although not showing any associations with muscle-related phenotypes, a positive effect on longevity was observed for the minor allele of rs1858828 in *SLC38A3* (OR=1.33 (1.06-1.68), p^{Add}=0.01). A lower chance to reach very advanced ages was found for the minor allele of rs12794763 in *SLC3A2* (OR=0.42, 0.26-0.68; p^{D}=0.0002), and for the minor alleles of three SNPs in *SLC38A9*: two of them, rs4865615 and rs7704138, are in LD with each other (r^2>0.8) and shows respectively OR=0.54 [(0.33-0.88), p^{Rec}=0.011] and OR=0.56 [(0.34-0.92), p^{Rec}=0.018]; rs10056358 shows a OR=0.66 [(0.45-0.96), p^{Dom}=0.029)].

Association with survival

Next, using 10-year of follow-up survival data, we investigated the association with survival of the analysed SNPs, estimating their quantitative effects. An increased risk of death was found for subject carriers of the C allele at rs14160 (*SLC36A1)* compared to those with the TT genotype (adjusted HR=1.59, 95% CI: 1.025–2.47; p=0.038) (Figure 1a). In *SLC38A9*, the presence of the A allele for the rs10056358 variation confers a survival advantage before the 90 years of age, compared to those with the TT genotype (adjusted HR=0.48, 95% CI 0.27-0.84, p=0.009) (Figure 1b), and a disadvantageous effect later, (adjusted HR = 1.43, 95% CI 1.01-2.03; p= 0.045) (Figure 1c).

Analysis of all other SNPs showed no statistically significant or suggestive differences in survival between genotypes.

Imputation of functional impact of SNPs

Finally, to explore the functional consequences of these polymorphisms we used regulatory information from ENCODE data using HaploReg v4.1 and RegulomeDB databases to investigate the possible functional impact of all the associated SNPs. Results from the functional annotation analysis are shown in Table S2. We found promising functional

implications for these SNPs (i.e. promoter and enhancer histone marks, transcription factor binding sites, and eQTL hits, indicating a regulatory potential. In particular, the functional implication of some SNPs is further corroborated by RegulomeDB results showing a score 1f (likely to affect binding and linked to expression of a gene target) for rs357629 (*SLC36A1*) and a score 2b (likely to affect binding) for rs4329925 and rs731710 (*SLC7A5*), and for rs1873793 (*SLC38A2*). LD patterns showed that all SNPs, except two (rs12794763, *SLC3A2* and rs999165, *SLC7A8*), were in LD with several variants that can collectively capture the casual variant.

Discussion

The goal of the current study was to investigate the impact of SNPs in selected AA transporters genes on physical performance and survival at old age.

Our analysis of a group of elderly individuals in the age ranges of 50-89 years, showed that genetic variants in *SLC7A5* (rs4329925) and *SLC7A8* (rs999165 and rs12588118), negatively affected HG strength, a reliable marker of the muscle performance and a predictor of disability and mortality in older adults [21]. Additionally, different SNPs in the same genes (rs731710 in *SLC7A5* and rs3783436 in *SLC7A8*) were significantly associated with ADL levels. *SLC7A5* and *SLC7A8* codify, respectively, for the plasma membrane proteins LAT1 and LAT2. Each of them forms with CD98 (*SLC3A2*) a heterodimeric bidirectional antiporter that regulates the simultaneous transport of leucine into cells and efflux of glutamine out of cells. Intriguingly, we found that two variants in *SLC3A2* (rs12804553 and rs4726) were associated, with opposite effects, with walking ability. These findings point to a role of the LAT1(LAT2)/CD98 complex in affecting muscle strength and physical performance in the elderly, likely through regulation of the anabolic signal mediated by the intracellular leucine/glutamine levels, and downstream activation of mTORC1. On the other hand, it is well known that leucine provides direct anabolic stimuli to skeletal muscle, so that the post-exercise leucine assumption is considered a potential treatment in numerous muscle wasting conditions [18]. As well, glutamine status is considered a hallmark of catabolic states and muscle loss [22].

Further supportive evidence to the above hypothesis is provided by the association with genes that mediate the transport of glutamine, such as *SLC38A2* (SNAT2) and *SLC38A7* (SNAT7). We found, indeed, significantly worse ADL scores associated to rs1873793 (*SLC38A2*), a finding supported by a disadvantage in attaining longevity for subjects carrying the same allelic variant, and a better walking performance associated to rs9806843 (*SLC38A7*). For the above transporters an important role in mTORC1 activation was documented. For instance, a reduction of SNAT2 activity in muscle cells is associated with reduced levels of glutamine and leucine and impaired protein synthesis through mTORC1 [23]; furthermore, it was found that the expression of system SNAT2, LAT1 and CD98 was upregulated after leucine availability in L6 myotubes [24]. The importance of these AA transporters is further bolstered by the fact that the heterodimer LAT1-CD98 also regulates the leucine flux into the lysosome [25], the major cellular compartment for mTORC1 activation [26], and that SNAT2 may act as a transceptor involved in both amino acid transport and signal transduction [27].

Although the mechanisms by which aging alters leucine-induced protein synthesis are currently under investigation, our data are in accordance with the hypothesis that a concerted activation of AA transporters may rely differently in young and older adults, finally influencing the anabolic response of skeletal muscle to AA availability. This hypothesis is supported by Drummond and Dickinson's work showing that aging differentially affected the expression of *SLC7A5* and *SLC38A2* in the skeletal muscle, in response to resistance exercise and essential AA supplementation [14, 17]. Also consistent with these evidences, are our findings regarding *SLC36A1* (PAT1), which has been reported to function as transceptors on the lysosomal membrane [28]. For this gene, we found the minor alleles at rs357618 and rs357629 (in LD with each other) associated with a better ADL performance. A different variant of *SLC36A1* (minor allele of rs14160), was found, instead, to increase the risk of death along a 10 year follow up of survival in our younger sample. A similar complex behaviour was observed for *SLC3A2*, with two SNPs conferring opposite effects on walking ability and another one negatively influencing the longevity phenotype. A possible explanation for these cross-phenotype associations would be that they represent pleiotropic effects of these transporters on different age-related phenotypes [29]. This could be in part related to the remodelling of physiological systems occurring with aging, a hypothesis supported by the observation that SNAT9 (*SLC38A9*), another lysosomal transceptor [30-32], shows a complex and dynamic association in this study. In fact, while a survival advantage was found for carriers of the rs10056358 A allele in the sample group aged 50-89 years, 90-plus subjects carrying this allele showed a lower probability to survive. Accordingly, the same minor allele A negatively influenced the probability to achieve longevity. Thus, this SNP manifests a survival trade-off, i.e. an antagonistic pleiotropic effect on survival at old ages, with the rs10056358-A allele conferring a positive effect on survival at ages before 90 and negative effect afterwards. The absence of correlation between *SLC38A9* gene and the parameters physical performance suggests that it functions as a pleiotropic gene with age-dependent effects, affecting survival and longevity but not quality of aging [33, 34]. On the contrary, conditional effects of these genes on quality of aging but not on longevity are shown for the most part of the genes studied in this work.

Overall, our data provide evidence that the genetic variability of these gene may impact on the muscle performance and/or the physical decline in adulthood, as well as on the probability to survive at old age. Thus, taking into account that AA transporters may act as sensors of AA availability and that this availability is closely related to skeletal muscle metabolism, AA transporter genetic variability may act at forefront of individual

susceptibility to anabolic resistance experienced with age, acting as risk factors for the onset of muscular decline in the elderly population.

Hence, understanding the functional implications of the associated SNPs may represent an important indication for elucidating the possible molecular mechanism underlying the associations found. However, the majority of the associated SNPs are non-coding variants, except two SNPs located in the coding region of respective genes. Our bioinformatics analyses identified, for some of them, a number of features (histone modifications, DNase I hypersensitivity clusters and transcription factor binding sites) consistent with a possible regulatory function, although we cannot exclude the possibility that the associated variants represent proxies for unknown causal variants as a result of LD, a part from those not showing any LD in their genomic region (rs12794763 and rs999165).

We are aware that our study has some weaknesses that should be addressed. A first limitation of the study is the lack of proper correction for multiple testing. Since this study was exploratory, a Bonferroni correction would have eliminated potentially important findings if applied. Another possible limitation could be the time of the survival follow up, not sufficient to draw long-term conclusions on the effect of genetic variants with minor effect on survival. Furthermore, the sample size could be increased and further explorations in additional study populations are needed before conclusions can be drawn.

Notwithstanding, and considering that this is the first study reporting genetic variants in AA transporter genes associated to the age-associated decline of muscle performance, we believe that our findings can open future investigations on the role of AA transporters in the quality of aging and longevity. This could provide valuable insights into potential targets for risk stratification in the population, and for therapeutic interventions aimed at increasing muscle mass and strength at old age.

Methods

Study population

The study was conducted on a sample of 729 subjects in the age range 50–107 years. The younger sample (age-range 50-89 years; mean age 70.42 ± 8.65) included 458 subjects (232 males and 226 females), the oldest old one (age range 90-108 years; mean age 97 ± 3.86) included 270 subjects (100 males and 170 females). See Table 1 for a complete description of the sample studied. All the subjects were born in Calabria (southern Italy) and their ancestry in the region had been ascertained up to the grandparents' generation. Samples were collected within the framework of several recruitment campaigns carried out for monitoring the quality of aging in the whole Calabria region from 2002 onwards. 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 through general physicians. Finally, each subject was 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. White blood cells (WBC) from blood buffy coats were used as source of DNA.

Ethic Statement

Recruitment campaigns and subsequent analyses received the approval of the relevant ethical committee. All the subjects provided written informed consent for the permission to collect blood samples and usage of register-based information for research purposes.

Physical Performance

Hand Grip (HG) strength was measured by a handheld dynamometer (SMEDLEY's dynamometer TTM) while the subject was sitting with the arm close to his/her body. The test was repeated three times with the stronger hand and the maximum of these values was considered 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. Since HG strength is affected by age, sex, and height, the scores were corrected for these factors.

Walking time was measured as the best performance (shortest time in seconds) of two walks along a 4-meter distance.

Functional Activity

The management of Activities of Daily Living (bathing, dressing, toileting, transfer from bed to chair, and feeding) was assessed using a modification of the Katz Index of ADL [35]. The assessment was based on what the subject was able to do at the time of the visit. The score is given counting the number of activities in which the participant is dependent or independent at the time of the visit. For the analyses, ADL scores were dichotomized as one if the subject was not independent in all five items and zero otherwise.

SNP selection and Genotyping

A total of 58 SNPs mapping within and nearby genes encoding 10AA transporters were prioritized by a tagging approach, attempting to choose those most likely to be of functional relevance (nonsynonymous SNPs, SNPs located in the 5′ and 3′ UTR regions). Supplementary Table S1 reports the complete list of selected SNPs, their position (relative to the chromosome and to the gene), and putative functional annotation.

Multiplex SNP genotyping was performed using PCR followed by primer extension and MALDI-TOF mass spectrometry using iPLEX Gold technology from Sequenom (Sequenom Inc, San Diego, USA). Sequenom MassARRAY Assay Designer software (version 3) was used to design primers for PCR and single base extension. Standard procedures were used to amplify PCR products, and unincorporated nucleotides were deactivated with the shrimp alkaline phosphatase (SAP). A primer extension reaction was implemented by mass extension primer and terminator. The primer extension products were then desalted on resin, and spotted onto the 384-element SpectroCHIP (Sequenom) for MALDI-TOF analysis using Spec-troACQUIRE v3.3.1.3 (Sequenom). Spectra were analyzed using MassARRAY Typer v3.4 Software (Sequenom). Approximately 10% of the samples were analysed in duplicate, and the concordance rate of the genotypes was higher than 99%.

Quality-control

After genotype calling, the dataset went through a battery of quality-control (QC) tests. At sample level, subjects with a proportion of missing genotypes higher than 10% were excluded from the study. At SNP level, SNPs were excluded if they had a significant deviation from Hardy-Weinberg equilibrium (HWE, $p<0.05$), a Missing Frequency (MiF) higher than 10% and a Minor Allele Frequency (MAF) lower than 5%.

Statistical analyses

Continuous and categorical variables were compared by using the independent samples t-test and the chi-square test as appropriate. For each SNP, allele and genotype frequencies were estimated by gene counting from the observed genotypes. Hardy Weinberg Equilibrium (HWE) was tested by Fisher's exact test. Pairwise measures of linkage disequilibrium (LD) between the analysed loci was estimated by Haploview (https://www.broadinstitute.org/haploview/haploview). Linear and logistic regression models were applied to estimate the impact of genetic variability on parameters of muscle strength (HG) and physical performance (WS and ADL), including as covariates age, gender, and height in the formulated regression models. A logistic regression model was also used to evaluate the effect of genetic variability on the chance to reach very advanced. In these models, genetic data was coded with respect to a dominant, recessive, and additive fashion. Then, for each SNP the most likely genetic model was estimated on the basis of minimum level of statistical significance (Wald' test p-value).

The hypothesis tested in this study is based on prior evidences for a role of AA transporters in the mTORC1-mediated induction of muscle protein synthesis. Furthermore, the involvement of genetic variability of AA transporters in relation to age-related muscle loss are lacking: in this sense, this study was exploratory. Thus, the p-values of single SNP analysis are reported without Bonferroni post hoc correction for multiple comparisons, to be not conservative and eliminate potentially important findings if applied.

In order to evaluate if the detected effects of the analysed polymorphisms on both muscle strength and physical performance might finally result in differential patterns of survival of the different relevant genotypes, we evaluated survival after 10 years from the baseline visit. Kaplan-Meier survival curves were estimated for each SNP affecting the analysed muscle-related phenotypes. In order to evaluate their predictive value with respect to mortality risk, the obtained survival curves were then compared by log-rank test. Subjects alive after the follow-up time were considered as censored, and this time was used as the censoring date in the survival analyses. In addition, Hazard ratios (HR) and 95% Confidence Intervals (95% CI) were estimated by using Cox proportional hazard models taking also into account possible confounder variables (age and gender).

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

Bio-informatic analyses

The functionality of theassociated SNPs was explored by retrieving regulatory information from the ENCODE (https://genome.ucsc.edu/ENCODE/) [36] and the Roadmap Epigenome Mapping projects (http://www.ppmroadmap.com/) [37] as implemented in HaploReg (v4.1, www.broadinstitute.org/mammals/haploreg/) [38], and RegulomeDB (www.regulomedb.org/) [39].

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Table 1: Socio-demographic characteristics and functional parameters in the sample stratified for group membership.

	50-89 (N = 475)	90-108 (N = 290)
Age (year)		
Mean (SD)	70.40 ± 8.711	96.86 ± 3.86
Male (%)	50%	37.24%
Height		
Mean (SD)	161.54 ± 9.28	151.39 ± 9.52
Range	138-190	125-175
BMI (SD)		
Mean (SD)	27.24 ± 4.19	23.12 ± 4.03
Range	17.80-45.35	12.98-40.54
HG strength		
Mean (SD)	22.86 (10.0)	-
Range	4-55	-
ADL* [n (%)]		
Non disable (=5)	84.4%	-
Disable (<5)	15.6%	-
Walking 4 meters [sec]		
Mean (SD)	7.77 (4.35)	-
Range	2.65-50.00	-

Abbreviations: BMI: Body mass index; ADL, Activity Daily Living; HG, Hand Grip Strength.

Table 2: SNPs showing at least one significant association with functional parameters under a nominal level (p Model < 0.05).

Gene	SNP	MAF	Hand Grip*		ADL°		Walking Time°	
			β ± se	pModel	OR (95% CI)	pModel	β ± se	pModel
SLC3A2 CD98	rs12804553	T=0.25					+2.48 ± 1.15	0.029[R]
	rs4726	T=0.20					-1.40 ± 0.68	0.034[D]
SLC7A5 LAT1	rs4329925	C=0.15	-1.98 ± 0.96	0.027[D]				
	rs731710	G=0.48			0.47 (0.27-0.81)	0.005[A]		
SLC7A8 LAT2	rs999165	A=0.19	-3.09 ± 0.95	0.0013[D]				
	rs12588118	G=0.27	-6.41 ± 2.06	0.002[R]				
	rs3783436	C=0.34			0.40 (0.20-0.82)	0.007[A]		
SLC36A1 PAT1	rs357618	G=0.30			0.11 (0.01-0.96)	0.013[R]		
	rs357629	G=0.32			0.11 (0.01-0.96)	0.013[R]		
SLC38A2 SNAT2	rs1873793	C=0.48			3.06 (1.36-6.85)	0.007[R]		
SLC38A7 SNAT7	rs9806843	G=0.34					+ 1.70 ± 0.63	0.008[D]

Abbreviations: MAF, Minor allele frequency; β, beta coefficient, se, standard error; OR, odds ratio; CI, confidence interval. p Model is the p value of the best genetic model, where R is recessive, D is dominant, and A is additive model.
*Age, sex, and height were included as covariates.
°Age and sex were included as covariates.

Table 3: Results of the association test with longevity in the analysed sample.

Gene	SNP	MAF	OR (CI)	pModel
SLC3A2 CD98	rs12794763	G=0.15	0.42 (0.26-0.68)	0.0002[D]
SLC38A2 SNAT2	rs1873793	C=0.48	0.70 (0.50-0.98)	0.035[D]
SLC38A3 SNAT3	rs1858828	T=0.38	1.33 (1.06-1.68)	0.014 [A]
SLC38A9 SNAT9	rs4865615	C=0.4	0.54 (0.33-0.88)	0.011[R]
	rs7704138	C=0.39	0.56 (0.34-0.92)	0.018[R]
	rs10056358	A=0.14	0.66 (0.45-0.96)	0.029 [D]

Abbreviations: MAF, Minor allele frequency; OR, odds ratio; CI, confidence interval. p Model is the p value of the best genetic model, where R is recessive, D is dominant, and A is additive model. The variable sex was considered a covariate.

Table S1 Description of the 58 SNPs initially selected.

Gene/ Protein	Chr	SNP	Position (GRCh38.p7)	Mutation type (Ensembl)	Major/Minor allele (Ensembl)	MAF
SLC38A3/ SNAT3	3	rs1858828	50204954	5'near gene variant	G/T	0.44
	3	rs2236939	50206077	Intron variant	G/T	0.11
	3	rs2236941	50210391	Intron variant	C/T	0.32
SLC36A1/ PAT1	5	rs3905908	151445768	5'near gene variant	T/C	0.22
	5	rs10077304	151452320	Intron variant	T/C	0.16
	5	rs918421	151452428	5'UTR variant	A/G	0.46
	5	rs11954054	151456584	Intron variant	A/G	0.23
	5	rs35060330	151458524	Intron variant	C/T	0.33
	5	rs17112008	151458557	Intron variant	C/A	0.33
	5	rs173349	151462020	Intron variant	T/C	0.47
	5	rs357616	151465808	Intron variant	T/C	0.40
	5	rs7730542	151466243	Intron variant	A/G	0.12
	5	rs357618	151467051	Intron variant	A/G	0.39
	5	rs357629	151471385	Intron variant	A/G	0.39
	5	rs14160	151491719	3'UTR variant	T/C	0.28
	5	rs1175	151492234	3'UTR variant	C/T	0.20
SLC38A9/ SNAT9	5	rs10056358	55630060	Intron variant	A/T	0.12
	5	rs2897834	55642751	Intron variant	C/A	0.13
	5	rs7704138	55648434	Intron variant	C/T	0.34
	5	rs10056287	55658650	Intron variant	T/C	0.19
	5	rs4865615	55664845	Missense variant	C/G	0.34
	5	rs7735053	55667626	Intron variant	T/C	0.34
	5	rs11749532	55706437	Intron variant	G/A	0.30
	5	rs7736177	55708116	Intron variant	G/A	0.31
SLC3A2/ CD98	11	rs12794763	62858040	Intron variant	T/G	0.15
	11	rs12221878	62858559	Intron variant	C/G	0.04
	11	rs10792362	62873879	Intron variant	T/C	0.43
	11	rs12804553	62876155	Intron variant	G/T	0.28
	11	rs4726	62885307	Exon Synonymous variant	C/T	0.25
	11	rs2282477	62889032	3'near gene variant	T/C	0.23
SLC43A1/ LAT3	11	rs2729385	57495520	Intron variant	G/A	0.33
	11	rs2584856	57499839	Intron variant	C/A	0.23
	11	rs61886993	57511522	Intron variant	C/G	0.08
	11	rs3851118	57513478	Intron variant	T/C	0.35
SLC38A2/ SNAT2	12	rs1873793	46367541	Intron variant	T/C	0.43
	12	rs2897968	46369903	Intron variant	A/G	0.36
SLC7A8/ LAT2	14	rs72684330	23124197	3'near gene variant	T/A	0.12
	14	rs17794251	23124233	3'near gene	C/T	0.27

				variant		
	14	rs3783436	23136764	Intron variant	T/C	0.34
	14	rs999165	23138657	Intron variant	T/A	0.27
	14	rs12588118	23147985	Intron variant	C/G	0.23
	14	rs10150592	23162327	Intron variant	C/A	0.17
	14	rs7141505	23183979	5'near gene variant	C/A	0.29
SLC7A5/ LAT1	16	rs4329925	87828401	3'near gene variant	T/C	0.14
	16	rs1060253	87832532	3'near gene variant	G/C	0.26
	16	rs9938601	87837658	Intron variant	A/G	0.42
	16	rs4843717	87844559	Intron variant	T/C	0.27
	16	rs731710	87850941	Intron variant	A/G	0.41
	16	rs7193392	87854200	Intron variant	G/A	0.39
SLC38A7/ SNAT7	16	rs9806843	58670957	Intron variant	A/G	0.42
	16	rs2280399	58677628	Intron variant	G/T	0.11
	16	rs7198514	58680599	Intron variant	C/T	0.20
	16	rs7187598	58681490	Non coding exon variant	T/C	0.41
	16	rs11640977	58681712	Splice region variant	T/C	0.14
	16	rs8058969	58685026	5'UTR variant	G/A	0.49
SLC1A5/ ASCT2	19	rs313835	46777532	Intron variant	C/T	0.28
	19	rs8105903	46784893	Intron variant	A/C	0.47
	19	rs1862336	46785388	5'UTR variant	T/C	0.24

Abbreviations: MAF (Minor Allele Frequency).
MAF refers to the European population as retrieved by Ensembl (www.ensembl.org)

Table S2. Summary of functional annotation of the phenotype-associated SNPs.

			HaploReg v4.1								RegulomeDB
GENE	**SNP**	**SNPs in LD (r^2≥0.8)**	**Promoter histone marks**	**Enhancer histone marks**	**DNase**	**Proteins bound**	**Motifs changed**	**GRASP QTL hits**	**Selected eQTL hits**	**dbSNP func annot**	**Score**
SLC3A2 CD98	rs12804553	4	SKIN	6 tissues	SKIN, PLCNT		4 altered motifs		8 hits	intronic	3a less likely to affect binding
	rs4726	8		15 tissues	7 tissues	POL2, POL24H8	Maf		7 hits	synonymous	4 Minimal binding evidence
	rs12794763	none	8 tissues	12 tissues			Foxl1, Pou1f1	1 hits	4hits	intronic	3a less likely to affect binding
SLC7A5 LAT1	rs4329925	31		12 tissues	13 tissues	POL24H8	Ets,RBP-Jkappa		1 hits	1.6kb 3' of SLC7A5	2b likely to affect binding
	rs731710	4		19 tissues	7 tissues		ATF3,ATF6,RFX5		3 hits	intronic	2b likely to affect binding
SLC7A8 LAT2	rs999165	none		8 tissues	GI,BLD		HDAC2,PRDM1,TATA			intronic	No Data
	rs12588118	8		13 tissues	4 tissues		4 altered motifs			intronic	4 Minimal binding evidence
	rs3783436	5		6 tissues	HRT,LIV		NF-Y	5 hits	5 hits	intronic	5 minimal binding evidence
SLC36A1 PAT1	rs357618	22					Egr-1		46 hits	intronic	6
	rs357629	22		FAT, SKIN	SKIN,SKN		Nkx3,Pou2f2	1 hits	46 hits	intronic	1f Likely to affect binding and linked to expression of a gene target

			HaploReg v4.1								RegulomeDB
GENE	**SNP**	**SNPs in LD ($r^2 \geq 0.8$)**	**Promoter histone marks**	**Enhancer histone marks**	**DNase**	**Proteins bound**	**Motifs changed**	**GRASP QTL hits**	**Selected eQTL hits**	**dbSNP func annot**	**Score**
SLC38A2 SNAT2	rs1873793	9	HRT	17 tissues	10 tissues	4 bound proteins	6 altered motifs	1 hits	7 hits	intronic	2b likely to affect binding
	rs1858828	14	10 tissues	17 tissues	5 tissues		BDP1,Ets, RXRA		41 hits	291bp 5' of SLC38A3	4 Minimal binding evidence
SLC38A7 SNAT7	rs9806843	2					FAC1,NF-I	1 hits	3 hits	intronic	4 Minimal binding evidence
SLC38A9 SNAT9	rs4865615	324		FAT, MUS			4 altered motifs		3 hits	missense	6 Minimal binding evidence
	rs7704138	318						1 hits	4 hits	intronic	5 minimal binding evidence
	rs10056358	11			4 tissues		Egr1, SETDB1	2 hits		intronic	4 Minimal binding evidence

Abbreviations: Promoter/Enhancer histone marks, regulatory chromatin states based on ENCODE and Epigenomics Roadmap data; DNase, DNase hypersensitivity based on Epigenomics Roadmap data; Proteins bound, proteins bound by chromatin immunoprecipitation based on Epigenomics Roadmap data; Motifs changed, altered regulatory motifs; GRASP QTL hits, quantitative trait loci based on GRASP (Genome-Wide Repository of Associations Between SNPs and Phenotypes); selected eQTL hits, expression quantitative trait loci based on the Genotype-Tissue Expression (GTEx) analysis.

Scores indicate the following degrees of evidence: Score 1a, eQTL + TF binding + matched TF motif + matched DNase Footprint + DNase peak; Score 2b, TF binding + any motif + DNase Footprint + DNase peak; Score 3a, TF binding + any motif + DNase peak; Score 4, TF binding + DNase peak; Score 5, TF binding or DNase peak; Score 6, other; "No data" indicates that RegulomeDB holds no information about the given SNP, meaning there currently exists no evidence to suggest that the SNP has a regulatory function.

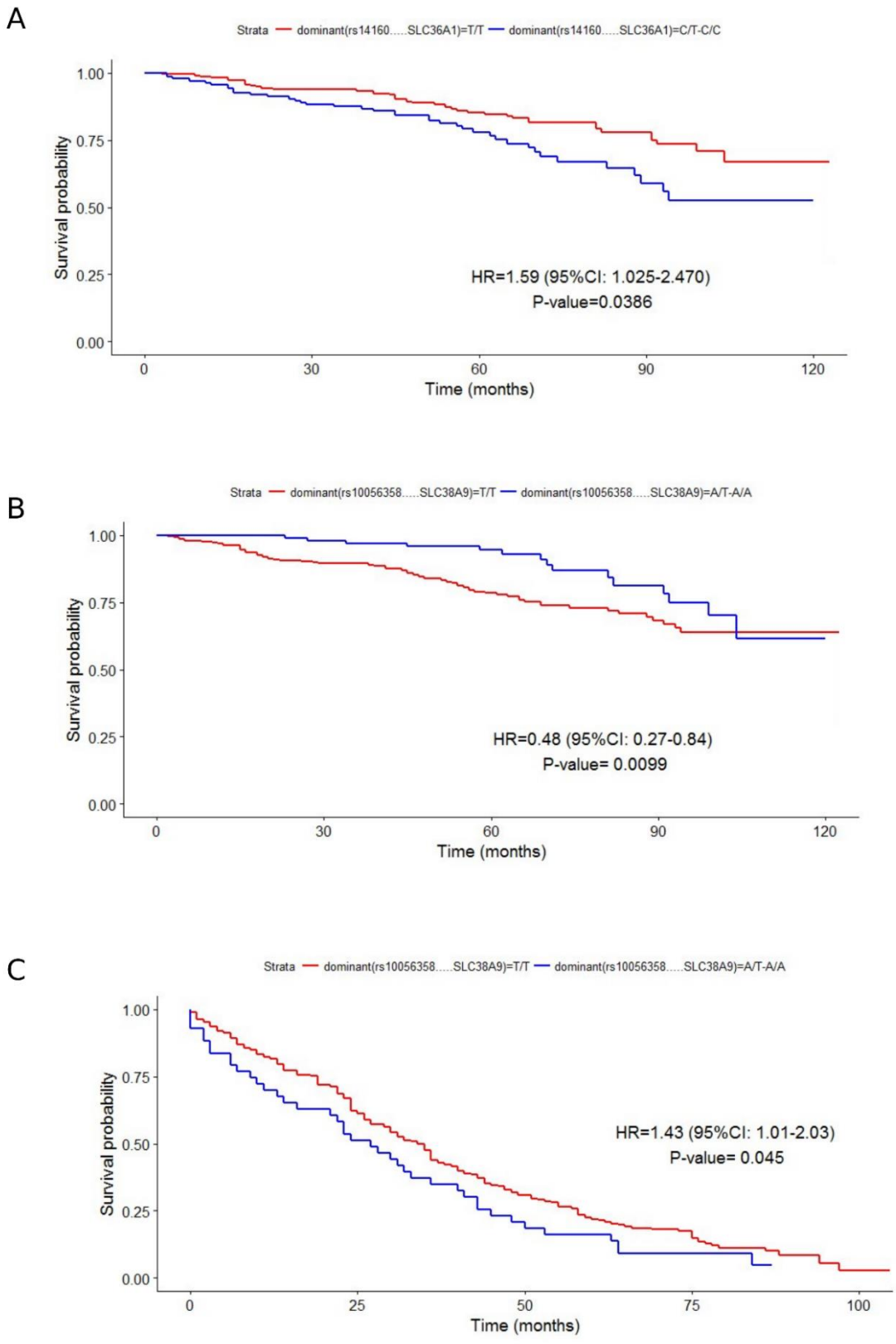

Figure 1: Survival function of carriers of minor allele (blue) vs non carriers (red). A) rs14160 (SLC36A1) in subjects aged <90 years; B) rs10056358 (SLC38A9)in subjects aged <90 years; C) rs10056358 (SLC38A9) in subjects aged > 90 years. Time is expressed in months, where 0 is considered the time of recruitment, and each individual is followed up for survival status till death. HR value, confidence interval and p-value from Cox regression analysis are reported inside the figure.

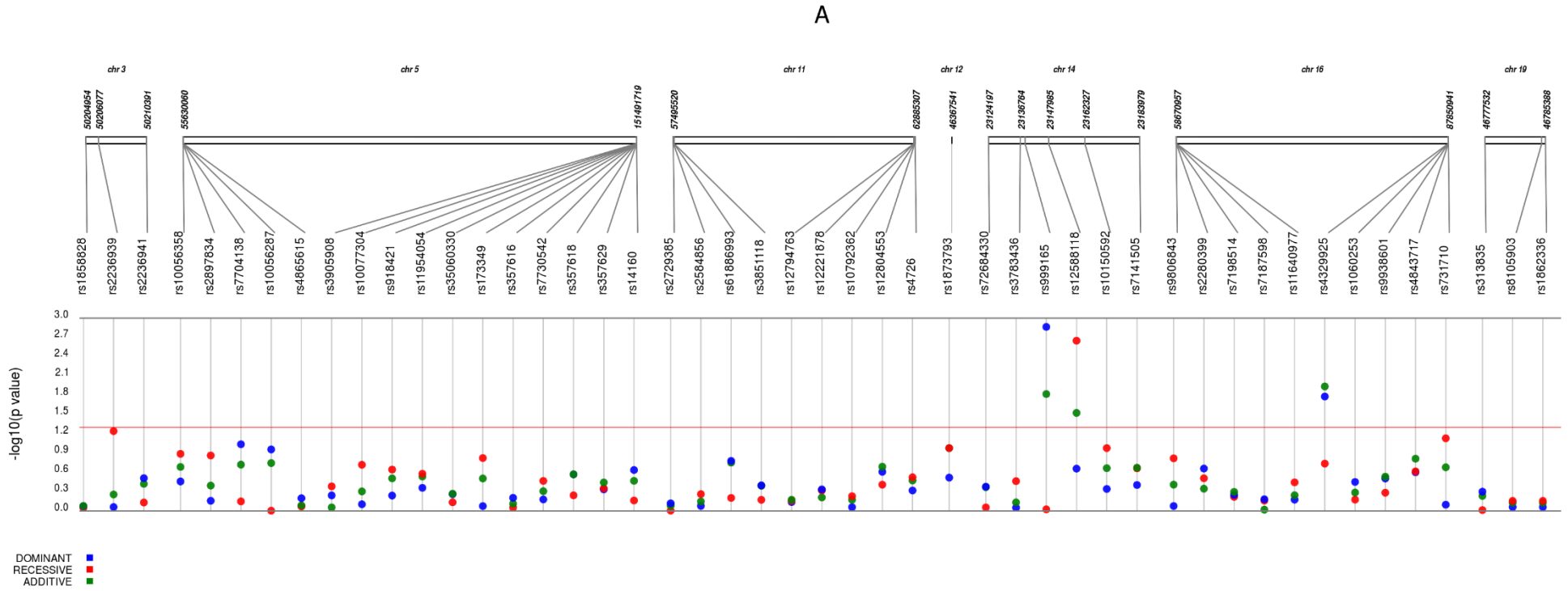

Figure S1 (a): Schematic representation of the association results with Hand Grip (HG) performance.

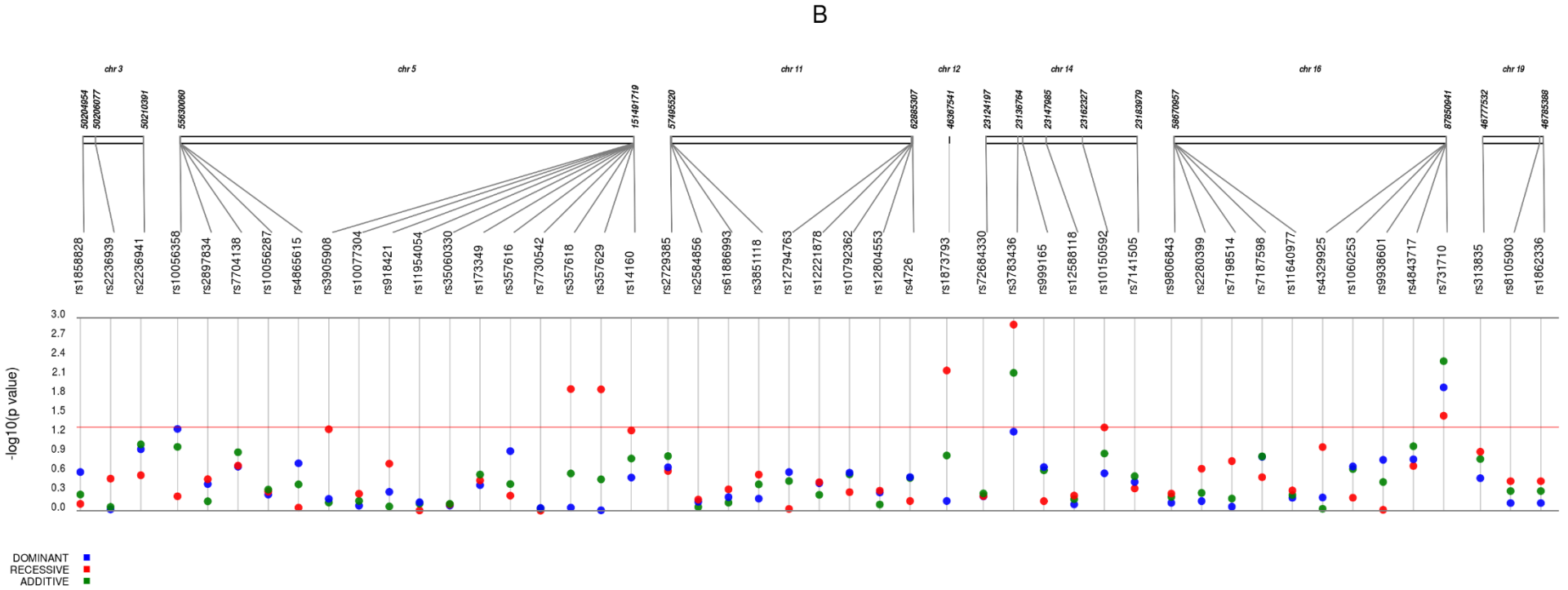

Figure S1 (b): Schematic representation of the association results with Activity of Daily Living (ADL) scores.

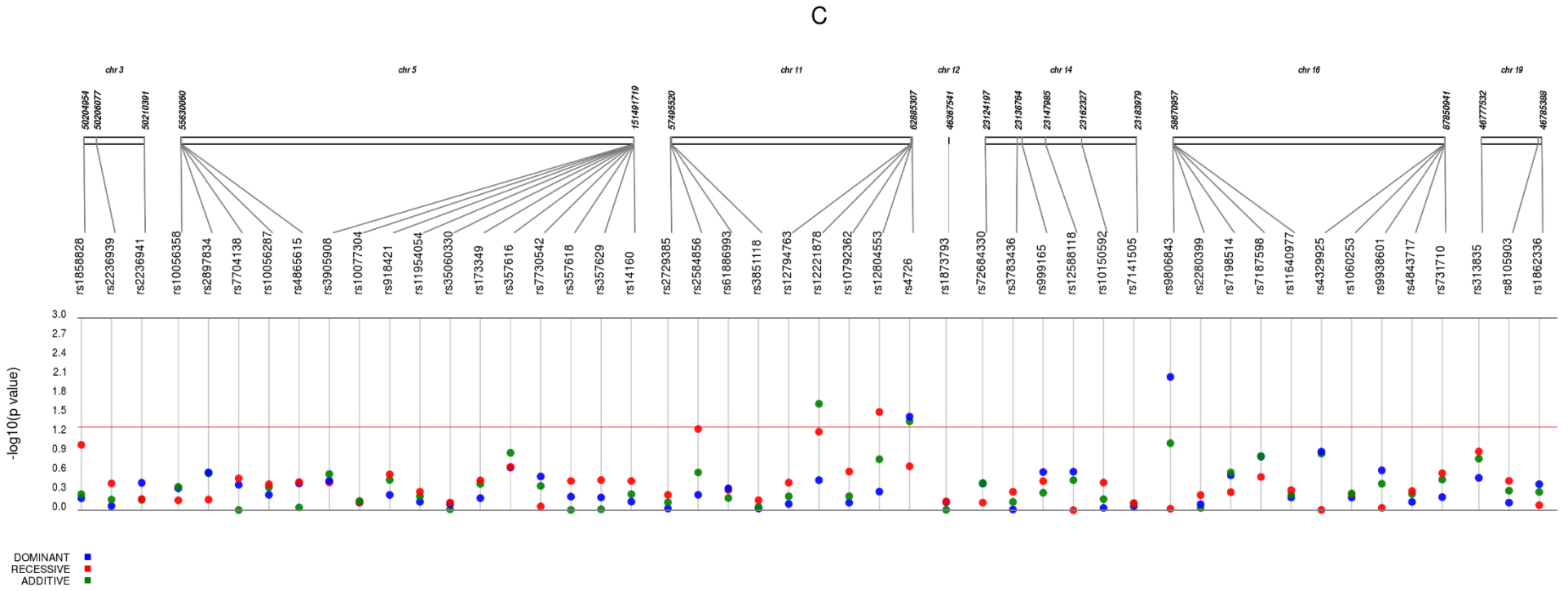

Figure S1 (c): Schematic representation of the association results with Walking Time (WT) scores.

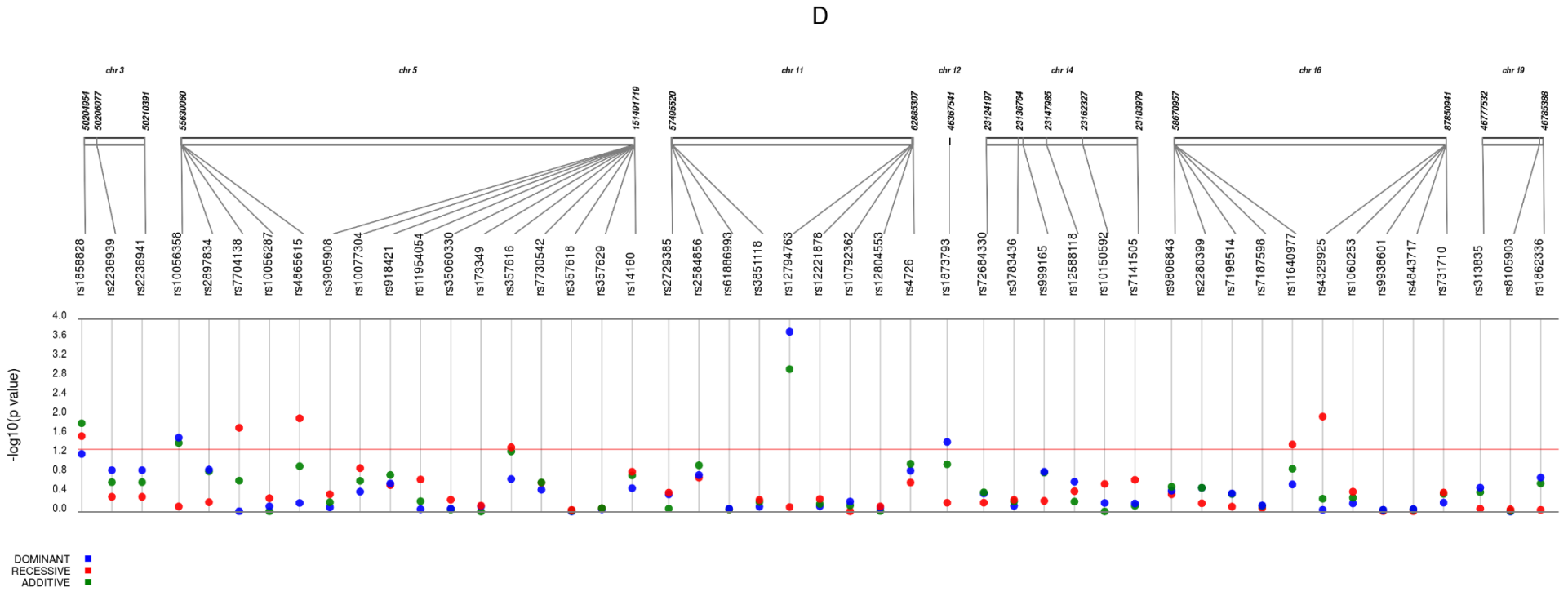

Figure S1 (d): Schematic representation of the association results with Longevity scores.

Chapter 7

End section.

7.1. Paper submitted to Advanced in Nutrition: Amino acids and amino acid sensing: implication for aging decline and diseases.

7.2. Short-term research program at University of Alabama in Birmingham.

7.3. Paper submitted to Aging Cell: Genotype- and age-specific effects of Lisinopril on mitochondrial biogenesis and function, reactive oxygen species levels, and metabolomic profiles in Drosophila melanogaster.

7.4. Paper published in DNA and Cell Biology: Reduction of Syndecan transcript levels in the insulin-producing cells alters glucose homeostasis in adult Drosophila melanogaster.

Amino acids and amino acid sensing: implication for aging decline and diseases

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Abstract

In humans, as in experimental models, gerontological researches indicate nutrition among the major modifiable determinants of healthy aging. Among nutrients, a direct impact on survival emerged in the last years for proteins and constitutive amino acids (AAs). Proof of this is the new concept of functional AAs, defined as those AAs able to regulate survival, growth, development, lactation, and reproduction of organisms, and whose deficiency impairs not only protein synthesis but whole-body homeostasis.

To shed a light on the role of AA sensing mechanisms and their modulation with aging, we reviewed the current knowledge about AA sensing, focusing our attention on mTORC1 and AA transporters as dynamic players of a complex network implicated in aging and age-related phenotypes. On the whole, by showing that alterations in AA sensors and signalling mediators are directly implicated in age-related phenotypes, literature supports the hypothesis that their modulation can represent a possible strategy for delaying aging decline. Thus, we suggest that the individual answer to AA availability may depend on the genetic background at components of sensing network and that the genetic screening in elderly population of variants involved in AA sensing pathways, analysed in combination with demographical and health status parameters, may help to clarify the role of AA sensing in lifespan regulation, finally opening new opportunities for therapeutic interventions.

Keywords: Amino acid availability, Amino acid sensing, Amino Acid Transporters, mTOR pathway, mTORC1, Nutrient sensing, Aging, Age-related diseases.

Introduction

The increase in life expectancy over the past century is an inevitable consequence of the demographic transition experienced by developed countries. This phenomenon has led to an "epidemiological transition", characterized by a marked reduction in the incidence of infectious diseases and the establishment of chronic degenerative illnesses (the so-called non-communicable diseases) (1), further contributing to increase the risk of developing disabilities and over time to the complete loss of autonomy. Much of the increase in chronic diseases associated with aging results from a changing in lifestyle, primarily in relation to feeding behaviour (increase in intake of foods high in saturated fat and low in unrefined carbohydrates) and decline in energy expenditure associated with sedentary habits.

The relationship existing between diet and human health is actually complex, considering that nutritional components have a regulatory role in physiological processes crucial for cell survival such as inflammation or immune function (2). On the other hand, dietary restriction (DR), namely the reduction of major dietary components (protein, lipid or carbohydrates) without malnutrition as well as the temporal variations of food intake (intermittent fasting), can provide higher health benefits, delaying the onset of many aging-associated pathologies (3).

In model organisms, many of the effects of DR on health and lifespan have been linked to reduced protein intake: in fact, protein restriction is able to decrease reactive oxygen species (ROS) production and macromolecules damages in Wistar rats, finally decreasing the aging rate (4). In humans, epidemiological data indicate a positive correlation between the high intake of animal-derived protein and adverse long-term side effects that manifest as chronic and age-related disease (5;6). For instance, follow-up studies on Europeans found that a high consumption of animal proteins leads to 75% increase in the overall mortality and fourfold to cancer in the age class 50-65 (7). At more advanced ages, high protein intake was associated with reduced overall mortality rate, probably due to a positive effect of protein consumption i.e. on muscle protein anabolism, which

help to avoid the progressive and generalised loss of skeletal muscle strength and mass experienced by the elderly (sarcopenia) (7). Thus, although evidences in humans and laboratory organisms indicate that dietary balance among nutrients has bigger effects on aging than individual components (8), a major concern of modern gerontology is the role of protein consumption for obtaining a healthy aging and longevity.

In the past years, it has become clear that AA availability is an important factor in the control of the rate of proteins' synthesis and catabolism and, therefore, affecting aspects of cell function, including regulation of cell signalling and gene expression as well as transport and metabolism of AAs themselves. For instance, while an increased AA availability stimulates protein synthesis, increases global mRNA abundance and inhibits protein degradation, under condition of scarce availability of AAs the above effects are reversed and catabolic processes such as autophagy and proteasome degradation are activated to maintain adequate AA levels (9). As well, AA sensing can have a central role in protein metabolism, with a direct impact on survival (10). At molecular level, AA sensing is mainly mediated by the mammalian target of rapamycin complex 1 (mTORC1) pathway and by the signal network initiated by the protein kinase called the General control non-derepressible 2 (GCN2). In recent years is emerging that also AA transporters play an important role in aa sensing, as they can function as transceptors, serving as or interacting with intracellular sensors which deliver information to signalling networks (11). To shed a light on the role of AA sensing mechanisms and their modulation with aging, here we review the impact of AA availability on health at advanced age, the molecular mechanisms of AAs sensing and how genetic modifications in components of AA sensing pathways may influence human aging and age-related diseases.

Current status of knowledge

Amino acid availability in aging and age-related phenotypes

Alongside being the constituents of proteins, AAs also represent building blocks for several other biosynthesis pathways necessary for growth and maintenance and play pivotal roles during signalling processes. Furthermore, they are key precursors of hormones and others substances of physiological importance, like glutathione and serotonin (12; 13). In mammals, nine out of the 20 amino acids are nutritionally essentials (EAAs; phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine), as they cannot be synthesized in adult humans. Among EAAs, branched chain amino acids (BCAA; isoleucine, leucine and valine) are key regulators of protein synthesis. Some nonessential AAs (NEAAs) can become conditionally essential in certain circumstances such as during periods of stress, aging, or illness (CEAAs; arginine, glycine, cysteine, glutamine, proline, serine, tyrosine).

Essential AA in aging and age-related phenotypes

For contrasting age-related phenotypes such as osteoporosis, frailty, sarcopenia or obesity and to promote health span, a central role is given to EAAs (14). Among them, a body of recent evidence suggests that in particular BCAAs behave as evolutionary conserved modulators of lifespan of different organisms, ranging from yeast to mammals (15). Nevertheless, evidences are contrasting: while the majority of authors found that BCAAs increase mitochondrial biogenesis and muscle function, others reported that an elevation in the level of BCAAs correlates with an increasing risk of insulin resistance (IR) and type 2 diabetes mellitus (T2DM) in humans and in rodent models (16; 17), finally predicting obesity and heart diseases. In mice, the specific restriction of dietary BCAAs determines weight loss, decreases fasting blood glucose levels, further improving glucose tolerance (18). A similar effect was observed in humans: subjects randomized to a low protein diet had significant decreases in fasting blood glucose and circulating levels of BCAAs, and experienced a significant loss of fat mass (18). In rats, meal distribution of leucine influences long-term muscle mass and body composition in adults (19) and decrease protein breakdown (20).

In humans, leucine provides direct anabolic stimuli to skeletal muscle (21), so that the post-exercise leucine assumption is considered a potential treatment in numerous muscle wasting conditions (22). In addition to favour muscle glucose uptake, leucine is able to increase fat leptin secretion and decrease food intake and body weight (15).

As for longevity, deprivation of some EEAs extends the lifespan across species: in particular, the selective restriction of methionine can mimic the effects of DR and extend life span through the activation of specific protective mechanisms such as the reduction of mitochondrial oxidative stress (23). In mice, leucine deprivation was observed to increase whole-body insulin sensitivity under insulin-resistant conditions (24).

Non-essential and conditional AAs in aging and age-related phenotypes

Among NEAAs, becoming CEAAs in periods of extreme trauma or stress, a prevalent role is reserved to glutamine, a key regulator of AA-controlled cell growth and the most abundant free AA in the blood (25). Recent evidences have highlighted non-anabolic functions of glutamine metabolism in regulating cell survival, oxidative stress resistance, signal transduction, and autophagy (26). For instance, glutamine is essential for the maintenance of normal neuronal physiology and skeletal muscle function due to its capability of controlling the heat shock response (27). Age-related changes in physiological systems (i.e. cardiovascular, endocrine, neuronal) and body composition may lead to lower availability of glutamine and chronic low-grade inflammation, consequently affecting its physiological roles. Thus, glutamine status may represent a hallmark of catabolic states: a low concentration of glutamine in plasma reflects reduced stores in muscle, particularly relevant in stress conditions. With aging, because of the physiological occurrence of muscle loss, glutamine deprivation can contribute to sarcopenia (27). In cancer, glutamine is a well-known nutrient effector of the metabolic program that fuels cancer cell growth and proliferation (26).

The CEAA arginine has been found to modulate the vascular inflammatory response, which in turn may result in a positive effect on vascular endothelial function. This is of clinical importance given that endothelial dysfunction is closely associated to the increased CVD risk associated with aging and to the age-associated declines in cognitive and physical functions (28). Finally, AAs availability is important also for maintaining an optimal brain function: among EAAs and CEAAs, aromatic (tryptophan, tyrosine, phenylalanine) and acidic (glutamate and aspartate) AA influences neurological functioning: the first are the biosynthetic precursors of the neurotransmitters serotonin, dopamine, and norepinephrine, while the second ones are themselves brain neurotransmitters (29).

Molecular mechanisms of AA sensing

How cells sense extracellular and intracellular AA levels represents a crucial node for an efficient regulation of downstream anabolic and catabolic effects of AA availability. Among the main mediators of AA sensing, a major role has been indicated for mTORC1, AA transporters and GCN2.

mTORC1 as AA sensor

mTORC1 is one of the two forms of mTOR, an evolutionarily conserved serine-threonine kinase of the superfamily of PI3KK (phosphatidylinositol-3 kinase related-kinases), that is at the centre of the signalling network that integrates the cellular response to different stimuli thereby regulating cellular metabolism and growth (Fig.1) (30).

FIGURE 1 IS ABOUT HERE

Pioneering work carried out in Xenopus oocytes and Chinese hamster ovary cells (31; 32) suggested that among the different AAs, mTORC1 responds to intracellular levels of leucine. Subsequent studies have highlighted sensitivities to other AAs, such as arginine, glutamine and serine (33-36).

Yet, how AAs availability is communicated to mTORC1 and how AAs modulate mTORC1 activity is only recently beginning to be understood. Figure 2 shows a schematic representation of the different components leading to mTORC1 activation by AAs.

In mammals, essential components in an AA-sensing pathway upstream of mTORC1 are four RAG (Recombination-Activating Gene) small GTPases, with RAGA or RAGB (RAGA/B) forming a heterodimer with RAGC or RAGD (RAGC/D) (37). The activation state of RAG GTPases is reflected by their guanine nucleotide binding status, and this is regulated by AAs. Under AA starvation conditions, the RAG GTPase complex is inactive and mTORC1 is dispersed in the cytoplasm. The presence of AAs promotes the formation of the active complex, in which RAG heterodimer contains GTP-bound RAGA/B and GDP- bound RAGC/D. The active RAG heterodimer binds directly to the mTORC1 component RAPTOR and redistributes mTORC1 to the surface of the lysosome where the small GTPase RHEB binds and activates mTORC1.

FIGURE 2 IS ABOUT HERE

It has been demonstrated that the Ragulator complex serves as a docking site for RAGs and mTORC1 on the lysosome, and functions as a guanine nucleotide exchange factor (GEF) for RAGA/B (38). In response to the presence of amino acids, Ragulator tethers RAGs to the lysosome, which in turn relocalize mTORC1 to this organelle leading to its activation. The vacuolar H+-ATPase (v-ATPase), an ATP-driven proton pump that maintains cytosolic pH by acidifying the lysosomal lumen, interacts with RAGs and Ragulator on the lysosomal membrane and this interaction is strengthened by AA starvation and weakened in response to AA stimulation. Thus, v-ATPase appears to act as un upstream regulator of mTORC1 in response to AAs. Zoncu et al (39) proposed a model in which the accumulation of AAs in the lysosomal lumen is sent to the Rag GTPases at the lysosomal surface through an inside-out mechanism.

Recently, proteins that sense specific AAs are reported to function as upstream regulators of the Rag GTPases and the mTORC1 pathway. In particular, as shown in Figure 2, Sestrin-2 protein is a specific sensor for leucine: in the absence of this AA, Sestrin-2 inhibits mTORC1 by interacting with GATOR2, which represses the GAP activity of GATOR1 toward Rag-A/B. Binding of leucine disrupts the Sestrin2–GATOR2 interaction and thus allows GATOR2 to promote mTORC1 activation via inhibition of GATOR1 activity (40).

It has been also demonstrated that the cytoplasmic protein CASTOR1 regulates mTORC1 in response to arginine. Under arginine deprivation, CASTOR1 forms a homodimer or a heterodimer with CASTOR2, and both complexes interact with GATOR2 to negatively regulate mTORC1 activity. Refeeding of arginine disrupts this interaction by binding directly to CASTOR1, thereby promoting mTORC1 activity (41).

Several others cytosolic AA sensors are involved in the activation of mTORC1, some of which directly bind AAs such as leucine [leucyl-tRNA synthetase (LRS), GDH (glutamate dehydrogenase), and UBR1-2 (unbranched chain AA receptors 1 and 2)], whereas others respond indirectly acting as cofactors to stimulate the activity of mTORC1 (11).

AA transporters as AA sensors

AA transporters are responsible for moving AAs in and out of a cell and between intracellular compartments. The transport mechanism may be facilitative (uniporter transporters) or may be coupled to movements of ions (Na+, H+, K+, Cl-), as well as movement of others AAs by symporter (coupled transporters) or antiporter (exchangers). On the basis of their substrates preference and sodium dependency, most of the AA transporters have been categorized into several groups defined as “System” (e.g, system A and N, or sodium dependent neutral AA transporters; System L, or sodium-independent large neutral AA transporters-), but they are currently grouped into solute carriers (SLC) families on the basis of sequence similarity. There are six major families of AAs transporters (SLC1, SLC6, SLC7, SLC36, SLC38, SLC43), and an “orphan” SLC16

(monocarboxylate family) transporter, which transports aromatic AAs. In addition to facilitate the delivery of AAs to the intracellular sensor molecules associated with mTORC1 and GCN pathways, new findings provide support for AA transporters as possible AA sensors. This role has been further supported by the evidence that they act as "transceptors", showing a dual transporter-receptor function, i.e, activation of an intracellular nutrient signalling cascade independent of AA transport, which enables them to sense extracellular AAs abundance (11;42).

As showed in Figure 3, leucine, glutamine and arginine are key stimulants of mTORC1 activation, thus suggesting a role of their transporters in the regulation of mTORC1.

FIGURE 3 IS ABOUT HERE

At the cell-surface, the transport of glutamine is mostly accomplished by system ASC AA transporter 2 (SLC1A5/ASCT2), while the influx of leucine is usually performed by system L AA transporter 1 (SLC7A5/LAT1). In particular, the complex SLC7A5/SLC3A2 (LAT1/CD98) regulates the simultaneous transport of glutamine out of cells and the transport of leucine into cells. Glutamine deficiency up-regulates the expression of SLC7A5 and other AA transporters causing a surge in the uptake of exogenous AAs, which in turn lead to mTORC1 activation (43). There is also evidence that inhibition of these transporters reduces mTORC1 signalling and prevents growth and proliferation of mammalian cells in culture (44-46). On the contrary, induction of SLC7A5 gene expression correlates directly with activation of mTORC1 (46; 47). Other cell-surface AA transporters (not showed in Figure 3), e.g, SLC7A8/LAT2, SLC43A1/LAT3, SLC43A2/LAT4, and SLC38A2/SNAT2 have also been linked to mTORC1 signalling (45; 48). As SLC7A5, the other three LAT family members are responsible for the majority of cellular leucine uptake. SLC7A8 requires the SLC3A2 co-transporter for function whereas SLC43A1 and SLC43A2 are monomeric facilitative uniporters. SLC38A2 transports small neutral AAs such as serine, alanine and glutamine, thus overlapping substrate specificity with SLC1A5. In response to extracellular AA

limitation, the expression of SLC38A2 is up-regulated by a mechanism dependent on the activating transcription factor 4 (ATF4), while the activity is increased by its enhanced stabilization (49). Additionally, SLC38A2 may activate mTORC1 through a signalling mechanism independent of intracellular AA concentration, thus acting as a putative AA transceptor (45).

AA transporters on lysosomal membranes also play a role in AAs sensing. Recent findings indicate that leucine flux into the lysosome is mediated by the heterodimer SLC7A5-SLC3A2, where it is recruited through association with the lysosomal-associated transmembrane protein 4b (LAPTM4b), leading to activation of mTORC1 (50). Two others AA transporters, the H+-coupled AA transporter SLC36A1/PAT1 and the SLC38A9/ SNAT9, have been reported to function as transceptors on the lysosomal membrane. SLC36A1, which is specific for alanine, proline, glycine, has both endosomal and plasma membrane localization and mediates AA efflux out of the lysosomal lumen, modulating the activity of mTORC1 (51; 52). However, when overexpressed in mammalian cell lines, SLC36A1 seems to have negative influence on lysosomal mTORC1 activation (39).

SLC38A9, which acts upstream of the Rag GTPases and Ragulator and in parallel with the v-ATPase, transports arginine across the lysosomal membrane, activating signal toward mTORC1 (34; 53). Gain of function of this transporter makes cells resistant to AA withdrawal, while loss of SLC38A9 expression impairs AA-induced mTORC1 activation, suggesting that SLC38A9 is an arginine sensor (54). Other lysosomal AA transporters implicated in mTORC1 activation include the histidine transporter SLC15A4 (55).

Recently, a member of the PAT/SLC36 family, SLC36A4/PAT4, has been shown to be mainly localised on the trans-Golgi network, where interacts with the small GTPase Rab1A to activate mTORC1, likely by sensing the intracellular levels of glutamine and serine (36; 56).

GCN2 as AA sensor

GCN2, a serine-threonine kinase, is another sensor of AA levels (57). Under conditions of AA starvation, uncharged tRNAs caused by the decline of AA concentration activate GCN2, which

phosphorylates the translational initiation factor eIF-2 α, thereby reducing the rate of protein synthesis. Additionally, activated GCN2 induces activity of ATF4, which up-regulates the expression of a spectrum of genes required for AA biosynthesis and import such as the SLC38A2 and SLC7A5 transporters (43; 58; 59). GCN2 has been connected to mTORC1 activation in some studies. For example, it has been proved that the activation of GCN2–ATF4 pathway in AA-starved cells leads to the inhibition of mTORC1 by transcriptionally up-regulating Sestrin2 (60). In particular, a more recent study demonstrated that GCN2 is involved in the inhibition of mTORC1 activity upon leucine or arginine deprivation through an ATF4 independent mechanism (61). Hence, activation of GCN2 and inhibition of mTORC1 act together to suppress the protein synthesis in condition of AA deficiency.

Genetic alterations in AA sensing in aging and age-related phenotypes

Alterations in components of mTORC1 complex

Genetic variations are widespread throughout *mTOR* gene and influence gene expression, finally affecting age-related phenotypes, although in recent years the most part of studies were focused on cancer susceptibility (62). Point mutations around the kinase domain of mTOR have been demonstrated to be associated with constitutive activation, cell cycle progression, and tumour cell size (62). To date, 20 of 129 common SNPs within *mTOR* have been investigated in relation to cancer risk, with seven polymorphisms (rs2536, rs2295080, rs1883965, rs1034528, rs17036508, rs3806317 and rs1064261) replicated in meta-analyses, and associated with susceptibility to different cancer types, from acute leukaemia to genitourinary cancers and oesophageal carcinoma (63; 64 and references therein). Polymorphisms either inducing up- or down-activity of mTOR may also play a role in other age-related diseases, such as cardiovascular and neurodegenerative disorders, still not investigated. Interestingly, some studies have found that mTOR inhibitors are able to prolong lifespan not just by inhibiting the growth of tumours, but by postponing the aging

process (65). It has been suggested that such an effect could be mediated by an attenuate protein aggregation, implicated in a wide number of age-related pathologies (66).

Genetic variants near the gene encoding for *MLST8*, a component of mTORC1 complex, positively correlated with the gene expression levels in several brain regions, were demonstrated to affect the "epigenetic age" of the cerebellum; furthermore, *MLST8* was found to overlap with signals from GWAS of various age-related diseases like those implicated in age-related macular degeneration, Alzheimer's disease and Parkinson's disease (67).

Alterations in AA transporters genes

In cancer cells, the expression of AA transporters, critical for control protein translation and cell growth through the mTORC1 pathway, support tumour metabolism (34). To date, the expression of several AA transporters has been associated to cancer (68). SLC7A5 up-regulation is commonly found in a wide spectrum of human cancers (69); consistently, its disruption by RNAi (70) or nucleases-mediated gene knockout (71) is able to reduce leucine uptake and cell proliferation. A role was established also for another transporter from LATs family, SLC43A1, originally identified as a cancer-up-regulated gene, regulated by androgens, and mainly detected in prostate cancer patients (69).

Behind a role in cancer development, these transporters may be implicated in other pathological phenotypes. For instance, SLC7A5 is also essential in maintaining normal levels of brain BCAAs. In mice, deletion of Slc7a5 from the endothelial cells of the blood brain barrier leads to unusual brain AA profile and neurological defects; moreover, homozygous mutations in SLC7A5 are associated with autistic traits and motor delay (72). Brain regions susceptible to neuronal loss as in Alzheimer's disease demonstrated greater expression of transcripts that reduced glutamate transport in vitro, in particular of SLC1A2, responsible for clearing the neurotransmitter from the synaptic cleft and performing 90% of glutamate uptake in mammalian central nervous system (73). Consistently, recent works found association of rare variation in the SLC1A2 with susceptibility to

bipolar disorder and schizophrenia (74). In hypertension condition, a role was suggested for sodium-dependent neutral amino acid transporter SLC6A19, which expression in rodent intestine depends on the presence of the carboxypeptidase angiotensin-converting enzyme 2 (ACE2): microsatellites in *SLC6A19* were found associated with hypertension, although most studies found differences in blood pressure only under stress-inducing conditions (75). SLC6A14 has a prevalent role also in obesity phenotype, through the potential regulation tryptophan availability for serotonin synthesis and thus possibly affecting appetite control. Genetic variation in the correspondent gene was associated to obesity in Finnish and French population with obesity (76; 77).

A crucial role in mediating T cell receptor (TCR)-stimulated glutamine uptake in naïve T cells has been reported for SLC1A5, which also regulates CD4+ T-cell differentiation and T cell-mediated immunity against infections and self-antigens (78).

Drummond et al (79) found that in skeletal muscle from young and elderly subjects, the expression of AA transporters like SLC7A5, SLC38A2, SLC3A2 and SLC36A1 is up-regulated after EAA ingestion likely through mTORC1 activation. Thereafter, Dickinson and co-workers demonstrated that their expression enhances after EAA ingestion and resistance exercise only in young individuals, suggesting that aging may influence the function of specific AA transporters, and possibly age-related phenotypes, such as sarcopenia (80).

Conclusions

Nowadays, nutrition, together with physical activity, is seen as a major modifiable determinant of chronic disease, with evidences increasingly indicating that diet has a huge impact, either positive or negative, on health during the whole lifetime, and that keeping a healthy diet and lifestyle can help to prevent and control morbidity and mortality resulting from chronic age-related diseases (81; 82). A major effect on health of protein consumption was indicated by the most recent lines of researches, with authors mainly agreeing that reducing protein intake during middle age followed by an increase from a moderate to high protein consumption in old adults may optimize health-span

and longevity (7). A growing body of literature focused its attention on a key role of AAs in health, prompting to a new concept of functional AAs, defined as those AA that regulate survival, growth, development, lactation, and reproduction of organisms (12). Thus, a deficiency of a functional AA (either EAA or NEAA) impairs not only protein synthesis but also whole-body homeostasis. On the other hand, the argument of AA supplementation is very discussed: while a positive effect on the preservation of lean body mass in chronic disease conditions such as cancer is recognised to BCAA supplementation (8; 83), epidemiological data collected in very large cohort of elderly people warn about supplementations, unable to promote a long-standing improvement of age-related conditions, and finally causing several drawbacks, such as nitrogen increase (84, 85).

In our opinion and considering the arguments discussed in this review, there are many remaining challenges ahead in our understanding of the role of AA availability in lifespan regulation. Firstly, the individual answer to AA availability may reasonably depend on the individual genetic background, i.e. polymorphisms in genes encoding for component of sensing network. These genes may influence health through pleiotropic effects at organismal level. Genetic screening in elderly population of variants involved in AA sensing, analysed in combination with demographical and health status parameters, may help to explain the age specific variability observed in relation to protein consumption. Then, network approaches, analysing whole pathways instead of single variants, may help to uncover additional players in this system, finely deciphering the molecular mechanisms involved in AA metabolism and possibly find new insights for understanding the consequences on health of their availability.

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Figure captions:

Figure 1: The structure of mTORC1 complex

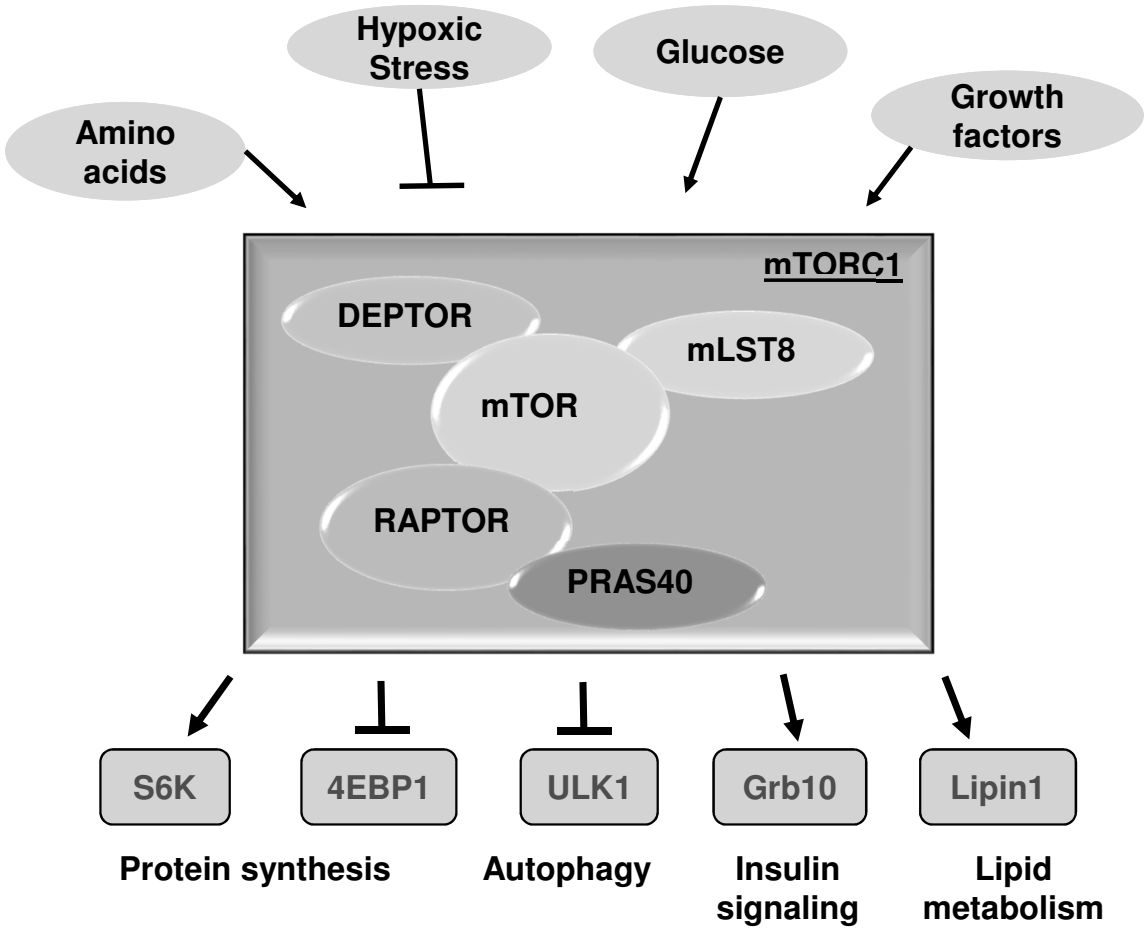
Schematic diagram of components mTORC1, its most well-known activators and the best characterized downstream targets.

Figure 2: Schematic model of mTORC1 activation by Amino Acids.

Amino acids within the lysosome generate a signal, which is communicated to the RAGs by the activation of the v-ATPase-Ragulator complex. Active RAGs recruit mTORC1 to the lysosomal surface, where it can be activated by Rheb, initiating mTORC1 signalling. Under amino acid deficiency, the inactive state of the v-ATPase-Ragulator complex is unable to activate Rag GTPases, thus mTORC1 cannot be recruited to the lysosome. AAs are also sensed outside the lysosome. The presence of leucine blocks the interaction of Sestrin2 with GATOR2, leading to the inhibition of GATOR1 and the activation of the Rag GTPases, allowing mTORC1 to be activated. CASTOR1 and CASTOR2 similarly regulate GATOR2 but are arginine dependent.

Figure 3: Amino Acids transporters and mTORC1 regulation.

Schematic model showing the integration of amino acid sensing by amino acid transporters and mTORC1 regulation. In different cellular compartments, specific transporters sense a specific amino acid content, and in collaboration with cytosolic amino acid sensors, regulate the activity of mTORC1. mTORC1 hubs controlled by SLC36A4-Rab1A appear to be Rag independent and not under the control of cytosolic sensors. At peroxisome-bound, TSC (tuberous sclerosis complex) blocks activation of mTORC1, but how this message is relayed remains unclear.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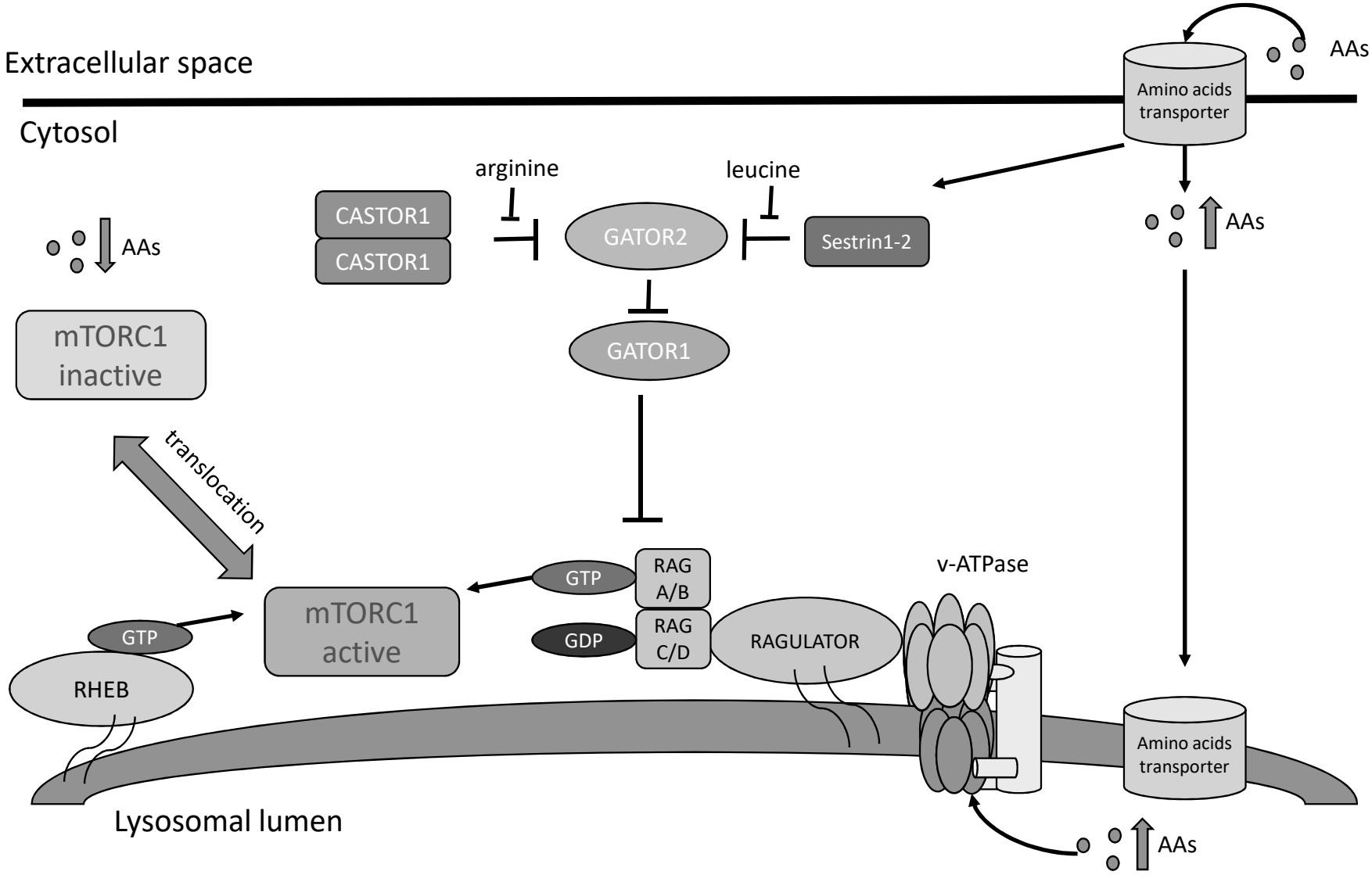
Extracellular space
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GATOR1
translocation
GTP
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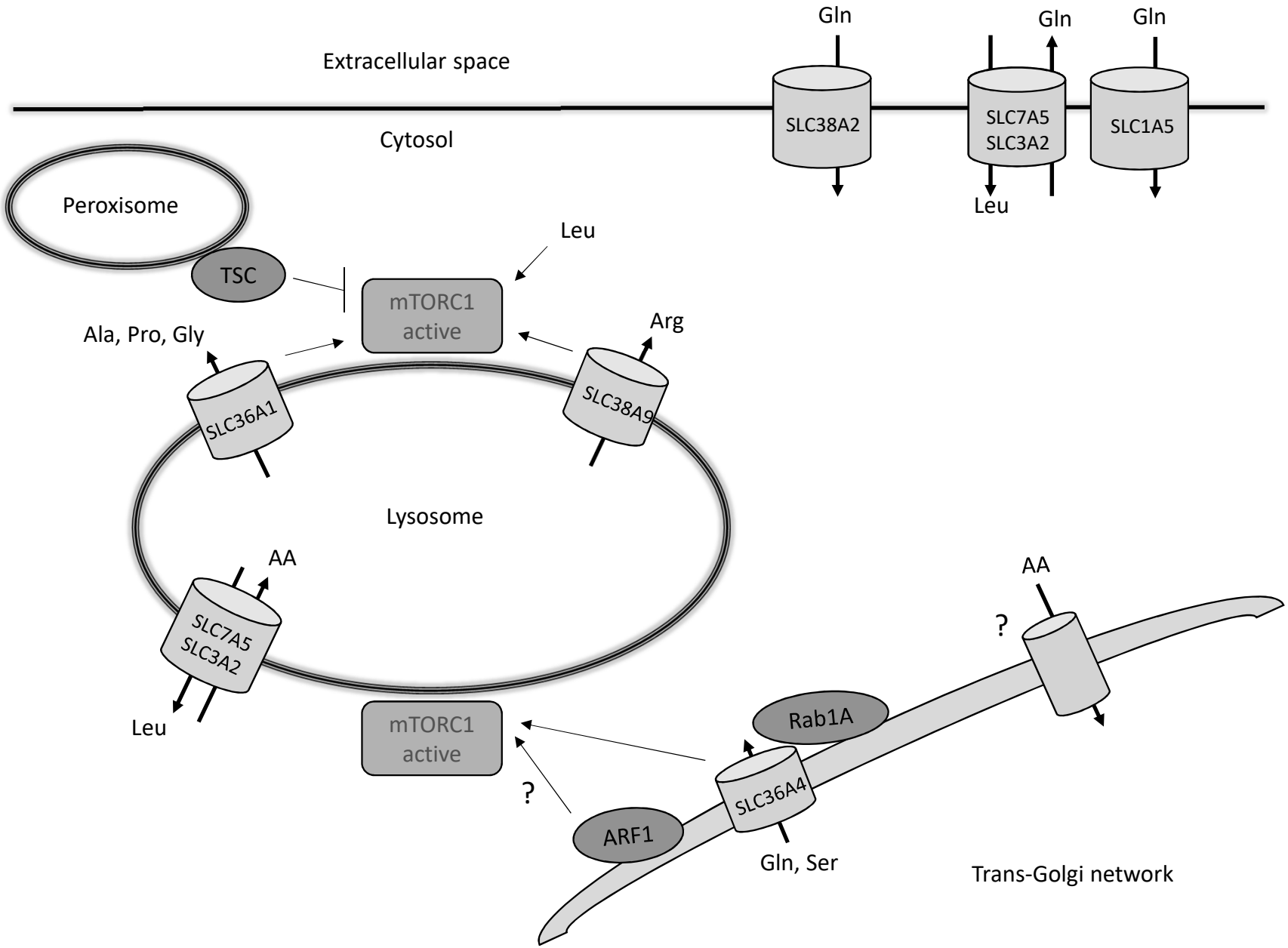
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Leu
mTORC1
active
Arg
Ala, Pro, Gly
SLC36A1
SLC38A9
Lysosome
AA
AA
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SLC7A5
SLC3A2
Leu
mTORC1
active
Rab1A
?
SLC36A4
ARF1
Gln, Ser
Trans-Golgi network

7.2. Short-term research program at University of Alabama in Birmingham.

A central goal of the newly established field of aging research termed "geroscience" is to unravel basic mechanisms of aging that drive multiple age-related chronic diseases in order to develop effective therapeutic strategies that enhance health span. For this reason and based on my research project I was involved in a 4-month research program in Doct. Maria De Luca laboratory, whose interest is focused on the identification of conserved genetic pathways that regulate metabolic homeostasis and lifespan using a quantitative genetic approach and Drosophila melanogaster as a model system.

Specifically, I had collaborated on a project about the role of ACEi therapy on longevity and on mithocondrial bioenergetics. It is well recognized that age-associated alterations in mitochondrial function and increased oxidative stress can have widespread effects on many cellular processes and have been implicated in pathological conditions as diverse as heart failure, hypoxia, diabetes, neurodegenerative disease and the physiological processes of aging. It has now become apparent that locally acting tissue renin-angiontensin system (RAS) also play a pivotal role in age-associated functional decline of organ and tissues. For example, elevation of angiotensin II often accompanies sarcopenia and cachexia, which are muscle wasting syndromes associated with aging and many chronic degenerative diseases. Pharmacological blockade of RAS by inhibition of the ACE has been demonstrated as an effective therapy in improving progressive cardiac dysfunction, renal disease, cognitive decline and impairment of physical function. Emerging evidence suggest that attenuation of ROS production and preserved mitochondrial function in energetically demanding organs and tissues, such as heart and skeletal muscle, provide some of the beneficial effects of RAS blockade.

To further understand the relationship between aging, ACEi and mitochondrial bioenergetics we had used the fly D. melanogaster, which is a well suited model system for investigating basic mechanisms of aging. The specific aims of this project were to determine whether genetic variation contributes to differences in variability in mitochondrial function and ROS levels in response to ACEi treatment across age groups and to identify novel genetic determinants of age-related changes in skeletal muscle mitochondrial bioenergetics in response to Lisinopril treatment (ACEi drug). The results of this study are reported in a paper whose manuscript has been submitted on Aging Cell attached above (De Luca M, Hoxha E, Wang L, Smith-Johnston KE, Jin K, Coward L, Gorman GS, Rowe G, Abadir P, Raftery D, Moellering D, Promislow D, Jumbo-Lucioni P - Genotype- and age-specific effects of

Lisinopril on mitochondrial biogenesis and function, reactive oxygen species levels, and metabolomic profiles in Drosophila melanogaster).

Furthermore, the main research interest of Doct. De Luca is the study of Syndecans (Sdc) proteins. Sdc are transmembrane heparan sulfate proteoglycans involved in multiple physiological processes, including the cellular response to heparin-binding growth factors, neuronal development, and inflammation. Sdc gene affects energy metabolism and lifespan in Drosophila melanogaster and genetic variants in the human SDC4 gene are associated with intra-abdominal fat, energy expenditure, and fasting plasma glucose levels in American children and with longevity in healthy elderly Italian subjects (Rose et al., 2015; De Luca et al., 2010). Functional follow-up studies in D. melanogaster, in which the Sdc gene was knocked down specifically in the fat body (the functional equivalent of mammalian adipose tissue and liver), revealed that fat body Sdc might play a role in a fuel-sensing mechanism that shifts resources to different physiological functions according to nutritional status in D. melanogaster (Eveland et al., 2016).

Moreover, syndecans in synergy with integrin-mediated signaling mediates cell adhesion to extracellular matrix. Engagement of extra cellular matrix (ECM) proteins to integrins and/or syndecans triggers mechanical and biochemical alterations, which finally leads to chromatin remodeling and gene expression changes (Nelson and Bissell, 2006). Several studies in humans and rodents strongly suggests that the protein composition and dynamic of ECM in several metabolic tissues play a critical role in the link between obesity and metabolic complications (Williams et al., 2015). However, we still know little about the impact of alterations in expression of mediators of cell-ECM interaction, such as syndecans, on the maintenance of energy balance and homeostasis. In mammals, the interaction between ECM components and their cell receptors has been shown to impact the transcription, secretion, and storage of insulin (Kaido et al., 2006). For this reason, we performed a study to determine whether knockdown of the Sdc gene expression specifically in the insulin-producing cells (IPCs) might affect insulin-like peptide (ILP) production and secretion. The results of this study are reported in the paper attached above (Warren JL, Hoxha E, Jumbo-Lucioni P, De Luca M – Reduction of syndecan transcript levels in the insulin-producing cells affects glucose homeostasis in adult Drosophila melanogaster. DNA and Cell Biology; 2017 Sep 25).

Genotype- and age-specific effects of Lisinopril on mitochondrial biogenesis and function, reactive oxygen species levels, and metabolomic profiles in *Drosophila melanogaster*

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Running Title: Lisinopril effects on *Drosophila* cellular energy metabolism

Summary

The angiotensin-converting enzyme (ACE) is a peptidase that is involved in the synthesis of Angiotensin II, the bioactive component of the renin-angiotensin system. A growing body of literature argues for a beneficial impact of ACE inhibitors (ACEi) on age-associated damage to various tissues/organs and on lifespan, mediated by cellular changes in reactive oxygen species (ROS) that improve mitochondrial function. However, our understanding of the relationship between ACEi therapy, mitochondrial biology, and aging is still limited. Here, we used genetically diverse strains *of Drosophila melanogaster* to test whether treatment with the ACEi Lisinopril differentially affects thoracic mitochondrial function and content and ROS levels at one week and three weeks of age. We then performed untargeted metabolomics analysis to pinpoint potential mechanisms underlying the effects of Lisinopril. We showed that Lisinopril treatment reduces thoracic mitochondrial respiration and ROS levels in young flies, and increases thoracic mitochondrial DNA content in older flies. However, these effects are genotype-specific, suggesting that Lisinopril might act through different mechanisms in different genetic backgrounds. Using metabolomics analysis, we identified 308 metabolite features with significant level changes after Lisinopril treatment. Metabolic pathways perturbed by Lisinopril are involved in glycogen degradation and glycolysis as well as mevalonate metabolism and triacylglycerol synthesis. We also found 37 metabolite features that vary significantly in response to Lisinopril in a genotype- and age-specific manner, including phospholipids and long-chain fatty acids. In conclusion, our results provide novel and important insights into the role of ACEi in cellular energy metabolism at different ages.

INTRODUCTION

Aging is a critical risk factor for most chronic diseases, which in turn are the major cause of disability and mortality worldwide (Niccoli & Partridge 2012). As such, current efforts to identify biomedical preventive strategies to slow basic biological processes of aging, such as mitochondrial dysfunction and oxidative stress, show great potential to promote increased healthspan (Kaeberlein *et al.* 2015; Longo *et al.* 2015).

Among the many hallmarks of human aging, we see an age-dependent steady increase in both systolic and diastolic blood pressure. This increase in blood pressure occurs in part as a result of chronic activation of the renin-angiotensin system (RAS) (Conti *et al.* 2012). The circulating RAS is a hormonal system whose primary function is to regulate arterial pressure as well as water and sodium homeostasis (Griendling *et al.* 1993). The main effector of RAS is Angiotensin (Ang) II, which is produced by enzymatic sequential cleavage of peptides derived from the liver-produced angiotensinogen. Angiotensinogen is converted by renin to Ang I, which in turn is converted to Ang II by the action of the angiotensin-converting enzyme (ACE) (Griendling *et al.* 1993). Ang II exerts its actions by binding with equal affinity to two main receptors, type 1 (AT_1) and type 2 (AT_2), which have opposite effects on vascular tone (Abadir 2011).

Within the past 15 years, however, it has become evident that, in addition to the circulating system, several RAS components are present in almost every organ (local RAS), where they exert diverse organ-specific physiological and pathophysiological functions through the action of Ang II (Benigni *et al.* 2010). Furthermore, growing evidence indicates that Ang II binding to the AT_1 receptor stimulates production of ROS via regulation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase activity (Nguyen Dinh Cat *et al.* 2013). Ang II- induced ROS, in turn, oxidize downstream redox-sensitive pathway targets involved in cellular processes, such as cell growth, inflammation, and fibrosis that promote tissue remodeling and repair (Vajapey *et al.* 2014).

Two drug classes that inhibit RAS by directly targeting Ang II, the ACE inhibitors (ACEi) and the angiotensin receptor blockers (ARBs), are widely used in clinical practice to manage cardio-vascular disorders and chronic kidney disease (Abadir 2011). However, pharmacological blockade of RAS, by ACEi or ARBs, has also been reported to favorably impact many chronic disease states affected by age as well as age-associated damage to various tissues and organ systems (Heudes *et al.* 1994; Basso *et al.* 2005; Abadir 2011; Simon *et al.* 2015). In addition, pharmacological and genetic studies in rodents and the worm *Caenorhabditis elegans* have revealed a role for RAS in the regulation of longevity (Benigni *et al.* 2009; Santos *et al.* 2009; Kumar *et al.* 2016).

Recently, several investigators have proposed that the beneficial effects of ACEi and ARBs on the age-related functional decline of organs and tissues are partly due to the capacity of these drugs to reduce ROS production and thereby preserve the physiological phosphorylation state of the mitochondria (Benigni *et al.* 2010; de Cavanagh *et al.* 2011; Conti *et al.* 2012). This idea is particularly intriguing considering clinical evidence that the renal and cardiac benefits of ACEi and ARBs in patients with hypertension and cardiovascular disease are somewhat independent from their blood pressure-lowering effects (Brenner *et al.* 2000; Viberti *et al.* 2002). However, disentangling the vascular hemodynamic effects of the drugs from their direct effects on cellular metabolism remains a challenge in humans and *in vivo* vertebrate models. To tackle this issue, in this study we used the invertebrate model *D. melanogaster*, which is an attractive model to study the relationship between ACEi therapy, mitochondrial function, and aging for several reasons. First, fly orthologues of human ACE, called angiotensin-converting enzyme (AnCE) and angiotensin-converting enzyme related (ACER), have been well described (Cornell *et al.* 1995; Taylor *et al.* 1996) and, like human ACE, regulate heart function (Crackower *et al.* 2002). Second, the activity of AnCE is inhibited by the same drugs (including Lisinopril) that inhibit human ACE through a similar mechanism (Kim *et al.* 2003). Third, mitochondrial morphology in *Drosophila* indirect flight muscles has shown to be a sensitive

pharmacological target of the ARB Losartan (Momota *et al.* 2013), suggesting a potential relationship between RAS-like components and muscle mitochondrial-related phenotypes in *Drosophila*.

A decline in thoracic mitochondrial function with aging, starting at relatively early ages (two to three-weeks), has been previously reported in *D. melanogaster* (Ferguson et al. 2005). However, we do not know whether the decline depends in part on genetic factors. Previously, we used 40 wild-derived inbred strains of the *Drosophila* Genetic Reference Panel (DGRP) to show that there is significant within-population genetic variability for mitochondrial function in the thoraces of young flies (Jumbo-Lucioni *et al.* 2012). Here, we used three additional DGRP strains (DGRP_229, DGRP_304, and DGRP_73) and a laboratory strain (w^{1118}) commonly used in genetic studies, to first measure thoracic mitochondrial function and ROS production between one-week and three-week old flies. The three DGRP strains were chosen because of their genetically-based differences in average lifespan (Durham *et al.* 2014). Subsequently, we treated young and older flies with the AnCE/ACE inhibitor Lisinopril to investigate the effect of the drug on thoracic mitochondria-related phenotypes and hydrogen peroxide (H_2O_2) levels. Lastly, we performed high-resolution global metabolite profiling in the thoraces of the three DGRP strains to identify metabolic pathways affected by the drug. We report that, as seen in mammals, ACEi administration affects thoracic mitochondrial function, mitochondrial content, and ROS production in *D. melanogaster*, strongly suggesting the existence of evolutionarily conserved physiological pathways linking ACEi and cellular energy metabolism. We reveal a novel metabolic signature of Lisinopril treatment. Furthermore, we show that Lisinopril effects on *Drosophila* cellular energy metabolism are strongly influenced by genetic background and aging, which therefore should be considered when AnCE/ACEi studies are designed.

RESULTS

Thoracic mitochondrial respiration declines in three-week old flies in a genotype-specific manner and independently of mitochondrial content

In this study we used the NAD^+-linked substrates pyruvate/proline to measure the oxygen consumption rate in the mitochondria isolated from the thoraces of one-week and three-week old flies, following methods described by (Jumbo-Lucioni *et al.* 2012). To analyze our data, we used a general linear model that allowed us to test for the main effects of genotype and age as well as the interaction effect between genotype and age. After adjusting for citrate synthase activity to control for differences in mitochondrial content, we found no significant main effect of genotype or age on state 3 respiration rate; however, there was a significant genotype-by-age interaction effect (Table S1, Supplementary information). State 3 respiration refers to the oxygen consumed in the presence of saturating amounts of respiratory substrate and ADP and is an index of oxidative phosphorylation (OxPhos) capacity. Mitochondria isolated from the thoraces of three-week old DGRP_229 and DGRP_73 flies had a significantly lower OxPhos capacity (57% and 74%, respectively) than those from one-week old flies. No age-related decline was seen in DGRP_304 or w^{1118} (Fig. 1A).

It is well established that mitochondrial coupling can be reduced by a basal leak of protons across the mitochondrial inner membrane (Jastroch *et al.* 2010). Given that basal proton leak is greatest under non-phosphorylating conditions (i.e., oxygen is consumed in the presence of respiratory substrate and absence of ADP) in isolated mitochondria (Jastroch *et al.* 2010), we assessed the mitochondrial basal state or state 2 and oligomycin-induced state 4 (state4o) respiration in the four *Drosophila* strains. We found that the age-dependent decrease in mitochondrial OxPhos capacity was accompanied by a significant reduction in both state 2 (55%) (Fig. 1B) and state 4o (59%) (Fig. 1C) respiration only in mitochondria isolated from the DGRP_73 flies (Table S1, Supplementary information).

To further corroborate that the age-related decline in mitochondrial respiration is independent of mitochondrial content, we measured the mitochondrial DNA/nuclear DNA (mtDNA/nDNA) ratio. Although we detected a significant difference in this ratio among the four strains (Fig. 1D), there was no effect of age (Fig. 1E) or genotype-by-age interaction (Fig. 1F) on mitochondrial content (Table S1, Supplementary information).

Thus, our findings confirm previous work in *D. melanogaster* showing a gradual decline in mitochondrial function, but they also suggest that genetic factors influence this decline. Indeed, while DGRP_73 flies showed an overall age-related decline in mitochondrial energetic capacity, DGRP_229 flies exhibited only a reduction in mitochondrial OxPhos capacity and DGRP_304 flies as well as the laboratory w^{1118} strain flies showed no changes in mitochondrial function at three weeks of age.

Three-week old flies from four genetically different strains have higher thoracic H_2O_2 levels than younger flies

An age-associated increase in production of cellular ROS has also been implicated as a putative mediator of the process of aging (Harman 1956) so we next assessed ROS production in our young and three-week old flies. Given that ROS, such as H_2O_2, are mainly produced by mitochondria but are also generated by other endogenous sources such as NADPH oxidase and xanthine oxidase (Harman 1956), we measured total thoracic H_2O_2 levels. We observed a significant difference in H_2O_2 levels among the four strains (Supplementary Table 2S), with both DGRP_229 and DGRP_304 strains having approximately 40% more H_2O_2 than DGRP_73 and w^{1118} (Fig. 2A and Table S2, Supplementary information) independently of aging. In addition, we found a significant increase (25%) in H_2O_2 levels in three-week old flies compared to younger flies (Fig 2B and Table S2, Supplementary information). Thus, an increase in H_2O_2 levels occurs among older flies, which appears to be genotype-independent.

Lisinopril treatment alters thoracic mitochondrial function, mtDNA content, and H_2O_2 levels in a genotype- and age-specific manner

Next, to test the idea that Lisinopril affects mitochondrial function, mitochondrial content, and ROS content in *D. melanogaster*, we fed newly eclosed male flies with food containing either 1 mM drug or no drug for one week or three weeks. The 1mM concentration is equivalent to the dose previously used by Momota and colleagues (Momota *et al.* 2013) to show a Losartan effect on muscle mitochondrial morphology. To confirm drug uptake we quantified Lisinopril concentrations in whole flies using liquid chromatograph-tandem mass spectrometry (LC-MS/MS). We observed the presence of Lisinopril in each strain; however, significant differences in Lisinopril concentrations among strains were also observed ($F_{3,27}$= 6.67, P = 0.002; Fig. 1S).

We used a general linear model to analyze our data and investigate the main effects of Lisinopril treatment, genotype, age, and all possible interaction terms on mitochondrial respiration, mitochondrial content, and H_2O_2 levels. We observed that Lisinopril significantly affected mitochondrial OxPhos capacity; however, this effect was influenced by its interaction with both genotype and age (*see* treatment-by-genotype-by-age interaction in Table 3S, Supplementary information). Unlike mitochondria isolated from DGRP_229, DGRP_304, and w^{1118} flies fed Lisinopril, those isolated from DGRP_73 flies consumed approximately 40% less oxygen during state 3 than untreated flies, but only at the younger age (Fig. 3A). Therefore, we assessed other mitochondrial function parameters in one-week old DGRP_73 flies and found that Lisinopril had no significant effect on mitochondrial state 2 respiration (Fig. 3B) or state 4o respiration (Fig. 3C). However, after measuring the enzymatic activity of three of the electron transport chain Complexes (I, IV, and V), we found a significant decrease (36%) in mitochondrial Complex IV activity with Lisinopril treatment (Fig. 3D). No differences were observed in the activity of Complex I (Fig. 3E) or Complex V (Fig. 3F).

Similar to mitochondrial respiration, there were significant Lisinopril-by-genotype-by-age interaction effects on both thoracic mtDNA copy number and H_2O_2 levels (Fig. 4A and 4B and Table 3S, Supplementary information, respectively) in the four *Drosophila* strains. DGRP_304 flies fed with Lisinopril displayed higher (22%) mtDNA content than DGRP_304 untreated flies, but only at three weeks of age (Fig. 4A). On the other hand, Lisinopril significantly reduced (50%) thoracic H_2O_2 levels only in DGRP_229 younger flies (Fig. 4B).

Taken together, these results suggest that Lisinopril alters mitochondrial OxPhos capacity and content as well as ROS production in *D. melanogaster*, but does so through different mechanisms that are influenced by genetic background and age.

Effects of Lisinopril on thoracic metabolomic profiles

Muscle is a highly plastic tissue. Pathophysiological and environmental perturbations lead to alterations in mitochondria bioenergetics and substrate metabolism in the muscle of diverse species, including *D. melanogaster* (Katewa *et al.* 2012). In this light, we investigated whether the effect of Lisinopril on thoracic mitochondrial function and ROS was accompanied by changes in the levels of two major thoracic metabolites, glycogen and triacyglycerol (TAG). We observed a significant effect of Lisinopril on glycogen levels but only in older DGRP_304 flies (Fig. 4C; Table 3S, Supplementary information). Along with higher levels of mtDNA content in their thoraces (Fig. 4A), three-week old DGRP_304 treated flies also stored more glycogen than untreated adults (Fig. 4C). On the other hand, a significant decrease in thoracic TAG levels was observed only in one-week old DGRP_229 and DGRP_73 treated flies compared to controls (Fig. 4D; Table S3, Supplementary information).

Motivated by these results, we performed high-resolution global metabolite profiling in the thoraces of the three DGRP strains to pinpoint potential mechanisms by which pharmacological blockade of AnCE influences thoracic metabolism. We detected 2674 and 1231 metabolite features in positive ionization mode and negative ionization mode, respectively

(Table S4, Supplementary information). We used linear models to analyze the data and identify individual metabolites showing changes in response to Lisinopril that (i) are independent from genotype and age (Lisinopril effect controlling for genotype and age effects), (ii) dependent only on genotype (Lisinopril-by-genotype interaction controlling for age effect), (iii) dependent only on age (Lisinopril-by-age interaction controlling for genotype effect), and (iv) dependent on genotype and age (Lisinopril-by-genotype-by-age interaction effects).

We found 308 features that showed significant changes in their levels after Lisinopril treatment (Table S5A). None of these metabolites vary significantly in response to Lisinopril in a genotype-specific or age-specific manner. To provide insight into the biological relevance of these metabolite features, we first performed a differential co-expression analysis and identified three and five modules (or clusters) of differentially co-regulated metabolites between Lisinopril-treated and control flies for negative and positive ion mode metabolites, respectively (Fig. 5A,B). We then ran the features from each identified module through the metabolite prediction program *Mummichog* (Li *et al.* 2013) to perform pathway enrichment analysis. Table S6, Supplementary information reports the full list of significant pathways in each module. Among the significant pathways, we observed enrichment for pathways related to glycogen degradation, glycolysis, methionine metabolism, and formyl tetrahydrofolate (THF) synthesis in the turquoise module for metabolites detected in negative mode (Fig. 5C). Further, the red module for metabolites detected in positive mode was enriched for pathways related to the mevalonate metabolic pathway and salvage of adenine and hypoxanthine (Fig. 5D). Additional pathway modules were related to TAG biosynthesis and the *de novo* biosynthesis of NAD from the amino acid tryptophan (see Table S6, Supplementary information).

Using our linear models, we also found 19, 1, and 37 metabolite features with levels that vary significantly in response to Lisinopril in a genotype-specific, age-specific, or genotype- and age-specific manner, respectively (Table S5B-S5D, Supplementary information). Metabolites perturbed by the drug in a genotype- and age-specific manner include 1-palmitoyl

lysophosphatidic acid, 3-hydroxy tetradecanoic acid (3-HTA), and DL-methionine sulfoxide (Fig. 2S; Table S5D, Supplementary information). We also observed that five of the thoracic metabolites that are affected by the Lisinopril treatment in a genotype-specific or genotype- and age-specific manner are phosphatidylethanolamines (PE) (Table S5B and S5D, Supplementary information). Furthermore, three metabolites, adenosine 5'-monophosphate (AMP), D-glucuronic acid, and D-glutamine, which are involved in glycolysis regulation, the glucoronate pathway and the tricarboxylic acid (TCA) cycle, respectively, showed genotype-specific changes in response to Lisinopril (Fig. 6; Table S5B, Supplementary information). While the Lisinopril treatment significantly increased the abundance of AMP (26%), D-glucuronic acid (97%), and D-glutamine (87%) in DGRP_229 flies, it significantly reduced D-glucuronic acid abundance (73%) in DGRP_73 flies. None of these metabolites appear to be affected by Lisinopril in DGRP_304 flies (Fig. 6).

DISCUSSION

Studies across a broad range of species have established a common set of evolutionarily conserved hallmarks of aging, including an age-related decline in mitochondrial function and increase in ROS production (Longo *et al.* 2015). This evidence points to the potential for pharmacological intervention to improve healthspan and extend longevity. To this end, strong evidence suggests that pharmacological inhibition of Ang II formation and action is not only beneficial in patients with hypertension, cardiovascular diseases, and diabetic nephropathy, but also displays age-retarding effects in humans and models systems (de Cavanagh *et al.* 2011). The mechanisms through which blockade of the bioactive component of RAS impacts the aging process and age-related diseases remain largerly unknown. However, there is a growing consensus that the beneficial effect of RAS blockade involves reduction in ROS production and thereby maintenance of mitochondrial function and content with advancing age (Benigni *et al.* 2010; de Cavanagh *et al.* 2011; Conti *et al.* 2012). In this study, we took advantage of the evolutionary conservation of ACE across species to study the effects of the ACEi Lisinopril on mitochondrial function and content and H_2O_2 levels in the thorax of the invertebrate model *D. melanogaster* at one week and three-weeks of age. We reasoned that the use of a model with an open circulatory system might provide important insights into the direct cellular effects of the drug.

The most important result of the present study is that Lisinopril treatment reduces thoracic mitochondrial respiration and H_2O_2 levels and enhances mitochondria content in a genotype- and age-specific manner. While the drug effects on mitochondrial respiration and H_2O_2 levels are observed in young flies of two different strains (DGRP_73 and DGRP_229, respectively), those on mitochondrial content are found in older flies of another strain (DGRP_304). Our study also showed that thoracic mitochondria of young DGRP_73 flies displayed a significant reduction in cytochrome c oxidase or complex IV activity upon Lisinopril treatment. Complex IV is one of the major regulation sites for O_2 consumption and, therefore,

could be responsible for the reduced respiration rate. Clearly, further research is needed to clarify the relationship. Of note, none of the Lisinopril effects were observed in flies from the w^{1118} laboratory inbred strain, which highlights the advantage of using wild-derived versus laboratory inbred strains for aging research. However, w^{1118} is also the strain with lower levels of Lisinopril concentration as assessed by mass spectrometry (Table S1). Thus, it is also possible that insufficient bioactive compounds reached the target site, which is an issue with any *in vivo* pharmacological investigation.

Previous work reported that AT_1 receptor knockout mice lived longer than controls (Benigni *et al.* 2009). Compared to wild-type mice, old mice deficient for the AT_1 receptor also showed a higher number of mitochondria (Conti *et al.* 2012). An increase in heart and liver mitochondrial number has also been observed in mice treated with the ACEi Enalapril from weaning until 24 months of age (de Cavanagh *et al.* 2011). In agreement with these observations, in our study Lisinopril treatment enhanced thoracic mitochondrial content and glycogen stores of three-week old DGRP_304 flies. This suggests that an increase in mitochondrial biogenesis could be the biological mechanism behind the longevity promoting effect of RAS blockade. Interestingly, proteins known to regulate mitochondrial biogenesis have been shown to be sensitive to changes in glycogen availability (Saleem *et al.* 2011). Whether the effect of Lisinopril on mitochondrial biogenesis, and potentially longevity, is secondary or not to its effects on glycogen homeostasis remains to be elucidated.

One important point is that although AnCE is evolutionary conserved, *Drosophila* does not have homologs of any other RAS components. Yet, findings in our study argue for the potential existence of a fly equivalent of the vertebrate Ang II/ AT_1 receptor system that is linked to mitochondrial biology and glycogen metabolism. This idea is strongly supported by previous work showing that administration of the ARB Losartan improved mitochondrial morphology in indirect flight muscles of *Drosophila* mutants of Multiplexin, the only orthologue of vertebrate collagen types XV and XVIII (Momota *et al.* 2013). Collagen types XV and XVIII are

proteoglycans present in the extracellular matrix (ECM) that bear glycosaminoglycan chains (Momota *et al.* 2013). An intermediate for the synthesis of glycosaminoglycan chains is D-glucuronic acid. D-glucuronic acid originates from UDP-glucuronic acid (http://www.hmdb.ca/metabolites/HMDB0000935), which is made from UDP-glucose, a precursor also for glycogen synthesis. We found that Lisinopril treatment increased the abundance of D-glucuronic acid as well as AMP in the thorax of DGRP_229 flies (Fig. 6). In mammals, regulation of glycogen metabolism is crucial in muscle energetics (Shulman & Rothman 2001), and AMP is required not only for activation of glycolytic enzymes (as shown in Fig. 6) but also of glycogen phosphorylase through its AMP-binding domain (Buchbinder & Fletterick 1996). As such, AMP promotes glycolysis and glycogenolysis, which in turn leads to the production of glucose 1-phosphate and its activation to form UDP-glucose and ultimately D-glucuronic acid (Fig. 6). Formation of the muscle-tendon interactions, in vertebrates and invertebrates, creates mechanical forces needed for the maturation of the myotendinous junction and differentiation of the tissue (Valdivia *et al.* 2017). This ECM remodeling of the junction is critical to protect against the load generated by muscle contraction (Valdivia *et al.* 2017) and an overlap between mechanisms regulating ECM remodeling and the breakdown of glycogen storage would therefore make biological sense. Given the extensive evidence that RAS blockade improves exercise capacity in elderly people (Simon *et al.* 2015), future studies addressing the hypothesis that the Ang II/ AT_1 receptor system might control mitochondrial biology, ECM remodeling, and glycogenolysis in skeletal muscle are warranted.

It is well recognized that mitochondria are gatekeepers for cell bioenergetics in most eukaryotic cells (Aon & Camara 2015). Cellular respiration is regulated by the need for ATP and the balance with other functions of the mitochondria. A pivotal role of mitochondria is in the regulation of cellular lipid homeostasis (Aon *et al.* 2014). This is due to the fact that mitochondria orchestrate the synthesis of key membrane phospholipids, such as PE, which in turn have many essential biological functions in cells (Vance & Tasseva 2013). PE are a class

of phospholipids, which together with phosphatidylinositol (PI) and phosphatidylserine (PS) moieties, form the backbone of most biological membranes of both eukaryotic and prokaryotic cells (Vance & Tasseva 2013). PE play a pivotal role in multiple cellular processes, including mitochondrial morphology and function (Vance & Tasseva 2013). For example, it has been reported that increased PE content induces autophagy and enhances longevity from yeast to mammals (Rockenfeller *et al.* 2015). On the other hand, depletion in mitochondrial content of PE affects mitochondrial fusion, mitochondrial ultrastructure, dynamics and function (Vance & Tasseva 2013). Increases in mitochondrial PE content and/or decreases in the molar ratio of PC/PE positively correlated to ATP content in mammalian hepatocytes and can modulate glucose production (van der Veen *et al.* 2014). It is, therefore, plausible that changes in the abundance of PE might in part explain the observed genotype-specific effects of Lisinopril on cellular metabolism.

The administration of combined drugs, such as statins and ACEi, is commonly used for the prevention and treatment of cardiovascular diseases due to their vasoprotective role (Faggiotto & Paoletti 1999). Studies in animal models suggest that statins and ACEi are strongly connected through the regulation of the mevalonate pathway, which is involved in the synthesis of cholesterol and is the best known target of statins (Cattaneo & Remuzzi 2005). *Drosophila* does not produce endogenous cholesterol, but statin treatment has been reported to increase the fly lifespan and improve cardiac health (Spindler *et al.* 2012). The identification of the mevalonate pathway (Fig. 5) as one of the metabolic pathways perturbed by Lisinopril in our study not only corroborates its mechanistic role in some of the additive effects of statins and ACEi, but also lays emphasis on other functions of the pathway.

In conclusion, our results provide novel and important insights into the role of ACEi in cellular energy metabolism and aging and establish *D. melanogaster* as a valuable model to better elucidate underlying mechanisms involved in the beneficial effects of these drugs on age-related decline in physiological functions.

MATERIALS AND METHODS

D. melanogaster strains and rearing conditions

Three wild-derived inbred DGRP strains, DGRP_73, DGRP_229 and DGRP_304, were obtained from the laboratory of Jeff Leips at UMBC. The laboratory inbred w^{1118} strain was obtained from the Bloomington Stock Center (stock number: 5905). Flies were reared in vials containing 10 ml of standard cornmeal, agar, molasses, and yeast medium, at a constant temperature of 25℃, 60–75% relative humidity, and 12hr/12hr light/dark cycle. Control groups were fed with standard medium and treatment groups received 1 mM Lisinopril (Sandoz Pharmaceuticals. Princeton, NJ) through its addition to the standard medium.

Mitochondrial function assays

Mitochondria isolation and respiration assays were performed as previously described in (Jumbo-Lucioni *et al.* 2012), with some modifications. Briefly, respiration rates were determined at 25℃ in respiration buffer (120mM KCl, 5mM KH2PO4, 3mM Hepes, 1mM MgCl2, and 0.2% BSA, pH 7.2) supplemented with 1mM EGTA, using Oroboros Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) with pyruvate 5mM/proline 5mM as complex I respiratory substrates. State 2 respiration was measured after addition of 1.3 mg of mitochondria and complex I substrates; state 3 respiration was induced by adding ADP (100 µM), and state 4 respiration was measured after adding oligomycin 16 µg/mL to inhibit ATP synthase. Mitochondrial loading was determined from protein content measured using the BioRad DC assay. Citrate synthase activity was measured as described in (Jumbo-Lucioni *et al.* 2012).

Mitochondrial complex activities were measured using standard spectrophotometric assays. Full details of metabolite detection and data analysis are reported in Supplemental methods, Supporting information.

mtDNA/nDNA ratio

Total DNA was isolated from 10 pooled thoraces using NaOH at 95℃ for 30 mins followed by neutralization with Tris-HCl. Quantitative PCR was performed in triplicate using

SYBR Green Master mix (Bio-Rad), primers for mitochondrial *16S rRNA* (F-AAAAAGATTGCGACCTCGAT; R- AAACCAACCTGGCTTACACC) and nuclear *RpL32* (F-AGGCCCAAGATCGTGAAGAA; R-TGTGCACCAGGAACTTCTTGAA) genes, on a 384 iCycler (Bio-Rad). mtDNA/nDNA ratio was calculated by comparative threshold method (Quiros *et al.* 2017).

H_2O_2 measurements

Five thoraces per genotype, age, and treatment were dissected between 10:00 AM and 11:00 AM from live flies in freshly prepared 20 mM N-ethyl maleimide, which is a cell permeable alkylating agent that prevents air-induced oxidation during fly dissection (Albrecht et al., 2011). Tissue H_2O_2 levels were quantified using the Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich#MAK165-1KT) according to the manufacturer's instructions. Fluorescence (λ_{ex} = 540/λ_{em} = 590 nm) was measured with a BioTek microplate reader (BioTek Instruments, Winooski, VT).

TAG and glycogen assays

Levels of TAG and glycogen levels were measured using previously reported protocols (Jumbo-Lucioni *et al.* 2010).

Statistical analysis

Mitochondrial respiration, complex V activity, and TAG and glycogen data were analyzed using ANCOVA (PROC GLM, SAS V9.3). We used the following mixed effect model: Y = cs (or pro) +strain + age + treatment + all interactions, where y = the trait value, cs or pro are the covariates citrate synthase activity or total protein levels, age and treatment are fixed effects, and strain is a random effect. Mitochondrial mtDNA/nDNA and H_2O_2 data were analyzed using ANOVA and the same model described above but without covariates. Reduced models without the treatment effect were run to test for the main effects of genotype and age as well as the interaction effect between genotype and age across the control groups. The Tukey test for *post hoc* pairwise was used to assess comparisons of differences among genotypes.

Comparisons for mitochondrial-related phenotypes between DGRP_73 Lisinopril treated and untreated flies were assessed by *t*-tests.

Global metabolomics profiling

Samples consisted of 36 thoraces (three genotypes, two ages, two treatments, and three replicates in each treatment/genotype/age group), which were flash frozen in liquid nitrogen between 10:00 AM and 11:00 AM in the De Luca lab, and then sent to the Northwest Metabolomics Research Center in Seattle, WA. Samples were thawed at room temperature, and then protein was precipitated using a cold methanol-water extraction, following previously described methods (Chiao *et al.* 2016). LC-MS analysis was performed using an LC-QTOF-MS system (Agilent Technologies, Santa Clara, CA) consisting of an Agilent 1200 SL liquid chromatography system coupled online with an Agilent 6520 time-of-flight mass spectrometer. LC-MS data was processed using Agilent Mass Profiler Professional (version 13.1.1) for compound identification. A list of ion intensities for each detected peak was generated using a retention time index and m/z data as the identifiers for each ion. Full details of metabolite detection and data analysis are reported in Supplemental methods, Supporting information.

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CONFLICT OF INTEREST

None declared

AUTHOR CONTRIBUTIONS

MD and PA conceived the study. MD, DP, DR, GSC, GR, and DRM participated in the design of the study and its coordination. MD, PJL, EH, KES, and LC performed research. MD, LW, and KJ analyzed the data. MD wrote the first draft of the manuscript. All authors read, critically revised the manuscript, and approved the final manuscript.

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SUPPORTING INFORMATION LISTING

Supplemental Methods. Liquid chromatograph-tandem mass spectrometry-based detection of Lisinopril, mitochondrial complex activities assays, and global metabolomic profiling.

Table S1. Mixed model ANOVA/ANCOVAs of thoracic mitochondria-related phenotypes.

Table S2. Mixed model ANOVA of thoracic H_2O_2 levels.

Table S3. Mixed model ANOVA/ANCOVAs of thoracic mitochondria-related phenotypes, H_2O_2 levels, and triacylglycerol and glycogen content in Lisinopril treated and control flies.

Table S4. List of metabolites detected by high-resolution LC-MS in the thoraces of three Drosophila Genome Reference Panel strains at one and three weeks of age.

Table S5. Untargeted metabolomics analysis results.

Table S6. *Mummichog* pathway enrichment analysis for modules of differentially co-regulated metabolite features between Lisinopril treated and control flies.

Figure S1. Lisinopril concentration in whole-body homogenates.

Figure S2. Metabolites with genotype- and age-specific changes in response to Lisinopril.

FIGURE LEGENDS

Figure 1. Thoracic mitochondrial respiration declines in three-week old flies in a genotype-specific manner and independently from mitochondrial content. (A-C) Significant genotype-by-age interaction effects on O_2 consumption of mitochondria isolated from thoraces in NAD^+-linked state 3 (Panel A; ANCOVA interaction term: $F_{3,43} = 14.92$, $p < 0.0001$), state 2 (Panel B; $F_{3,43} = 7.61$, $p = 0.0005$), and state 4o (Panel C; $F_{3,43} = 14.92$, $p < 0.0001$) respiration rate. Boxes indicate least-square (LS) means adjusted for citrate synthase activity from $n = 7$ independent replicates. Error bars represent standard errors. (D-F) Differences in mitochondrial content are explained by genotype (Panel D; $F_{3,35} = 174.01$, $p = 0.0007$), but not age (Panel E) or age-by-genotype interaction (Panel F) effects. Box and whiskers plots denote individual data points separated by a line representing the group median. Each individual value is plotted as a dot superimposed on the boxplots. In all panels, *p<0.05, ***p<0.001, and ****p<0.0001, obtained from Tukey *post hoc* tests for multiple comparisons.

Figure 2. Three-week old flies from four genetically different strains have higher thoracic H_2O_2 levels than younger flies. (A-B) Differences in H_2O_2 levels are explained by genotype (Panel A; ANOVA: $F_{3,68} = 14.15$, $p = 0.0282$) and age (Panel B; $F_{1,68} = 10.27$, $p = 0.0478$) effects**.** Box and whiskers plots denote individual data points separated by a line representing the group median. Each individual value is plotted as a dot superimposed on the boxplots. In both panels, *p<0.05, ****p<0.0001, obtained from Tukey *post hoc* tests for multiple comparisons.

Figure 3. Effects of Lisinopril on thoracic mitochondrial function depend on genotype and age. (A) Significant Lisinopril-by-genotype-by-age interaction effects on O_2 consumption of mitochondria isolated from thoraces in NAD^+-linked state 3 (ANCOVA interaction term: $F_{3,84} = 2.70$, $p = 0.0509$). Boxes indicate least-square (LS) means adjusted for citrate synthase activity from $n = 7$ independent replicates. **p<0.01, obtained from Tukey *post hoc* tests for multiple comparisons. Error bars represent standard errors. (B-C) The effect of Lisinopril on

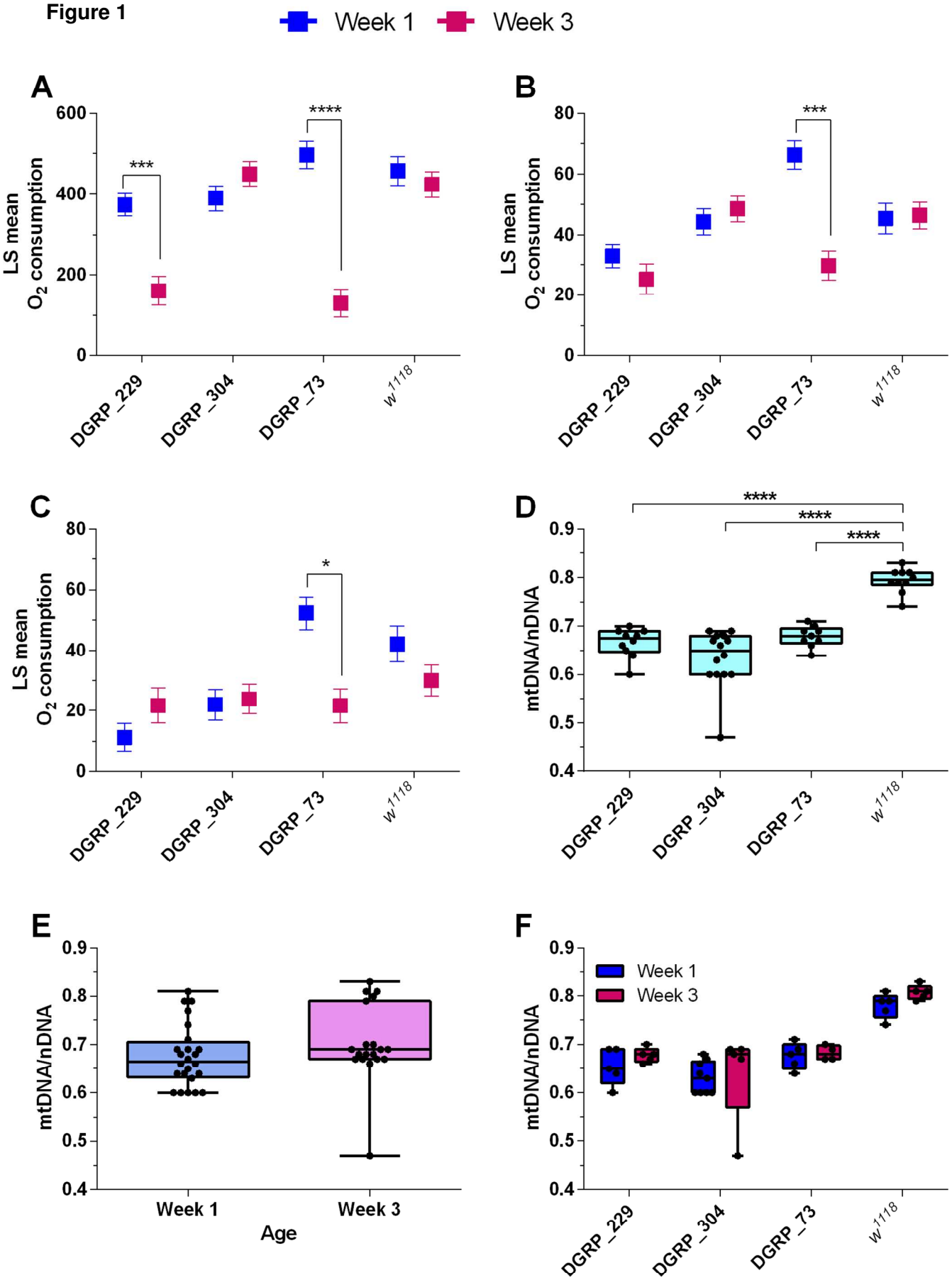
mitochondrial OxPhos capacity in young DGRP_73 files is not accompanied by a change in state 2 (Panel B) or state 4o (Panel C) respiration rate. (D-E) Lisinopril reduces the activity of mitochondrial Complex IV (Panel E), but not Complex I (Panel F) or Complex IV (Panel G), in young DGRP_73 flies. In Panels B-G, box and whiskers plots denote individual data points separated by a line representing the group median. Each individual value is plotted as a dot superimposed on the boxplots. **p<0.01 for comparisons between treatment means.

Figure 4. Lisinopril increases thoracic mitochondrial DNA content and glycogen and reduces levels of H_2O_2 and TAG in a genotype- and age-specific manner. (A-B) Significant genotype-by-age interaction effects on mitochondrial DNA content (Panel A; ANOVA interaction term: $F_{3,67} = 4.73$, p =0.0047) and tissue H_2O_2 levels (Panel B; ANOVA interaction term: $F_{3,147} = 3.06$, p <0.03). Box and whiskers plots denote individual data points separated by a line representing the group median. Each individual value is plotted as a dot superimposed on the boxplots. (C-D) Significant genotype-by-age interaction effects on glycogen (Panel C; ANCOVA interaction term: $F_{3,98} = 3.55$, p =0.0173) and TAG levels (Panel D; ANCOVA interaction term: $F_{3,136} = 3.84$, p = 0.0111). Data represent least-square (LS) glycogen and TAG means adjusted for total protein content from *n* = 5-8 and *n* = 8-10 independent replicates, respectively. Error bars represent standard errors. In all panels, **p<0.01, ***p<0.001, and ****p<0.0001, obtained from Tukey *post hoc* tests for multiple comparisons.

Figure 5. **Significant modules and functionally enriched pathways involving differential thoracic metabolites between Lisinopril treated and control flies**. (A and B) Heat maps of correlated metabolite features detected in negative ion mode (Panel A; 3 modules) and positive ion mode (Panel B; 5 modules). Each point represents the correlation between two metabolite features and the color scale bar indicates the value of the correlations. (C and D) Representative pathways in the turquoise module for metabolite features detected in negative mode (Panel C) and in the red module for metabolite features detected in positive mode (Panel D). Numbers in parentheses indicate overlap size/pathway size.

Figure 6. Three metabolites with genotype-specific changes in response to Lisinopril are involved in glycolysis regulation, the glucoronate pathway, and the tricarboxylic acid (TCA) cycle. Data reported on the plots represent the mean l*og2* abundance of three replicate samples for each treatment and genotype group. *p<0.05, **p<0.05, ***p<0.001 after Benjamini and Hochberg's adjustment for multiple comparisons. Error bars represent the 95% confidence interval.

Figure 1


Week 1
Week 3
A
B
C
D
E
F
LS mean
O_2 consumption
mtDNA/nDNA
DGRP_229
DGRP_304
DGRP_73
w^{1118}
Week 1
Week 3
Age
***
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*

Figure 2

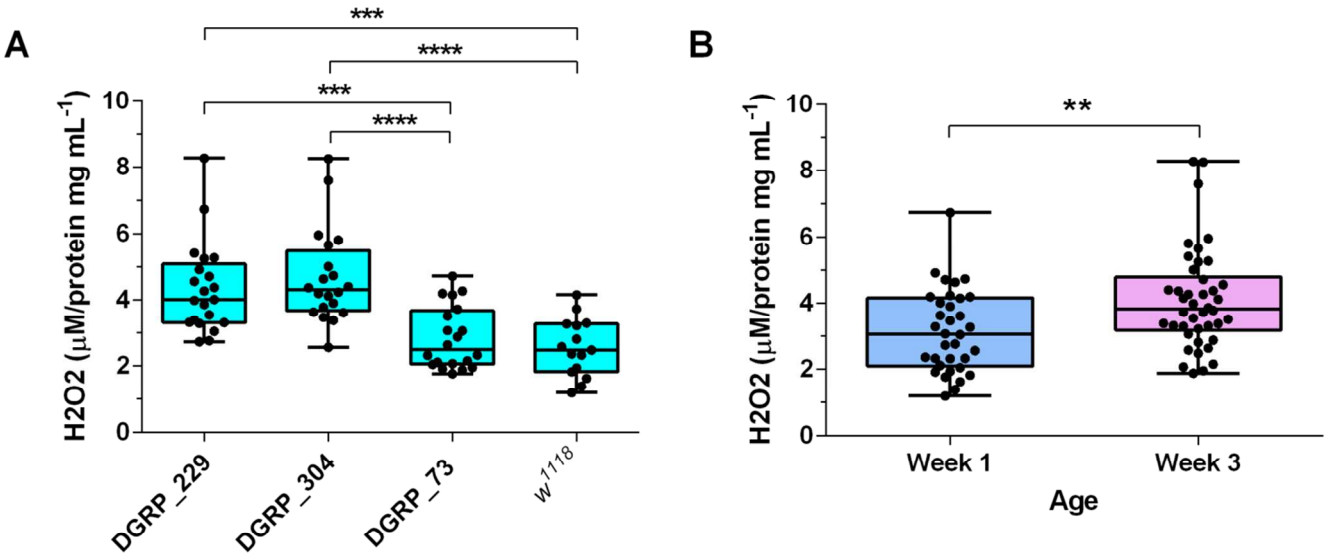


Figure 3

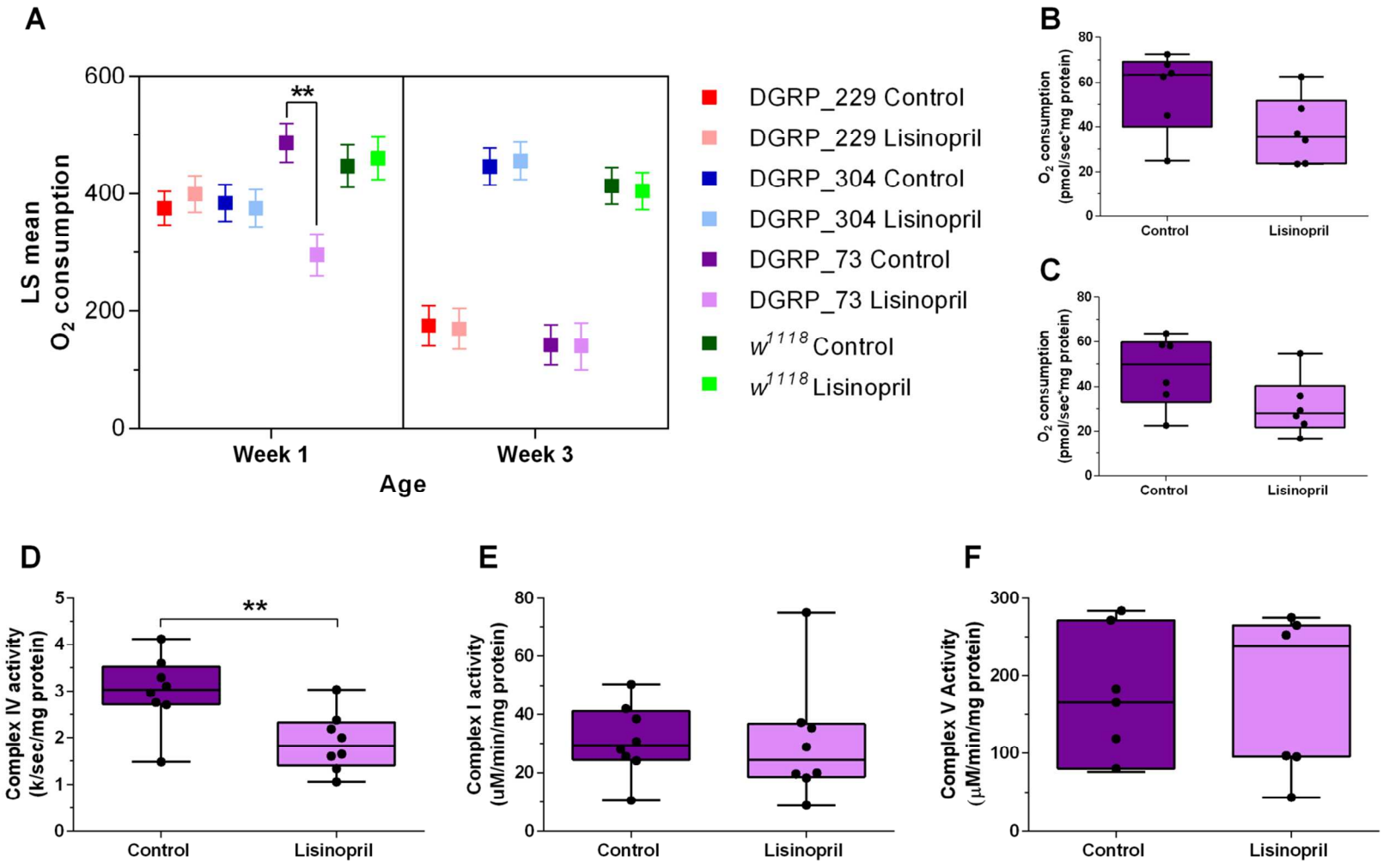

A
600
400
200
0
**
LS mean
O2 consumption
Week 1
Week 3
Age
DGRP_229 Control
DGRP_229 Lisinopril
DGRP_304 Control
DGRP_304 Lisinopril
DGRP_73 Control
DGRP_73 Lisinopril
w1118 Control
w1118 Lisinopril
B
O2 consumption (pmol/sec*mg protein)
Control
Lisinopril
C
O2 consumption (pmol/sec*mg protein)
Control
Lisinopril
D
Complex IV activity (k/sec/mg protein)
**
Control
Lisinopril
E
Complex I activity (uM/min/mg protein)
Control
Lisinopril
F
Complex V Activity (μM/min/mg protein)
Control
Lisinopril

Figure 4

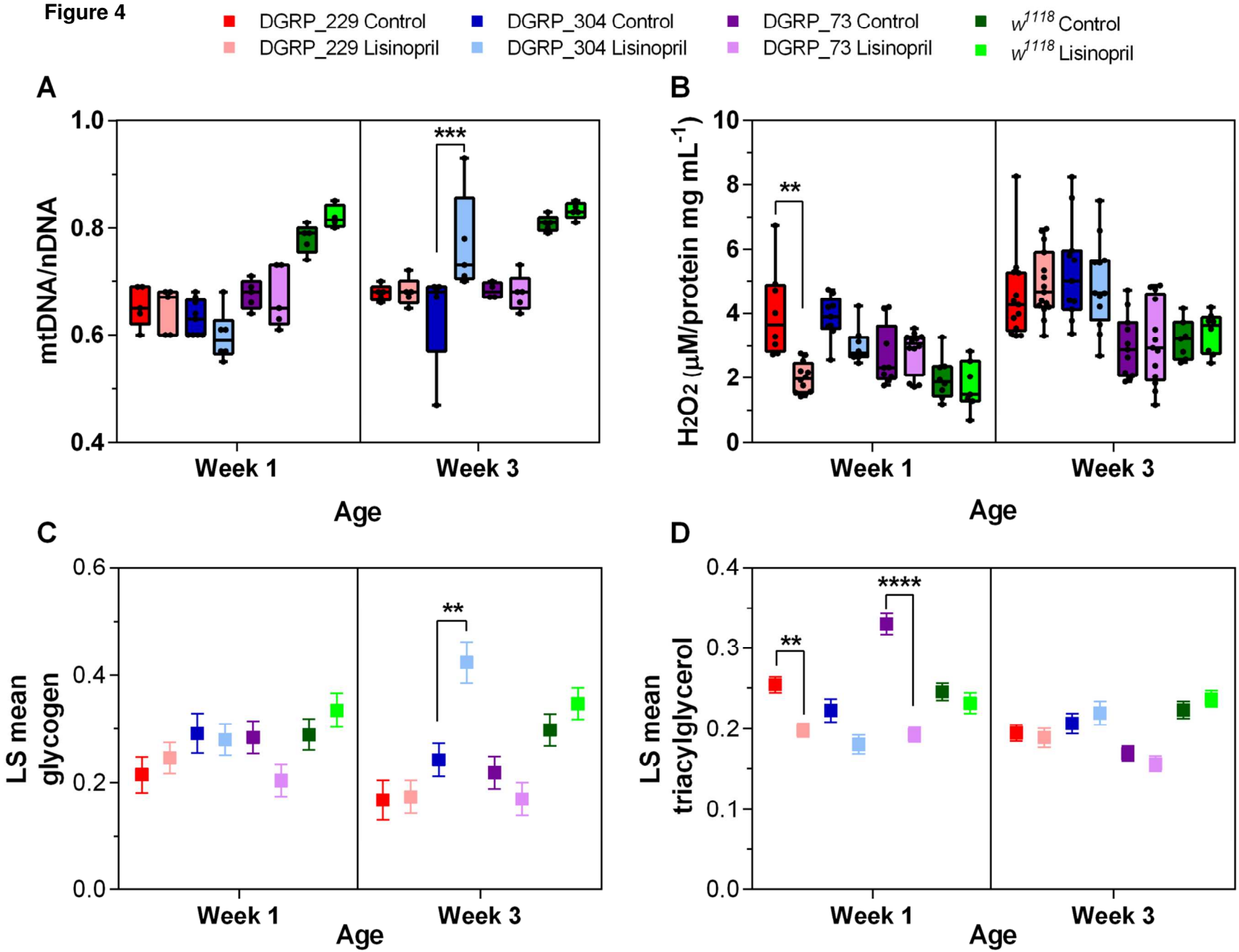

DGRP_229 Control
DGRP_229 Lisinopril
DGRP_304 Control
DGRP_304 Lisinopril
DGRP_73 Control
DGRP_73 Lisinopril
w1118 Control
w1118 Lisinopril
A
mtDNA/nDNA
1.0
0.8
0.6
0.4
***
Week 1
Week 3
Age
B
H2O2 (µM/protein mg mL-1)
10
8
6
4
2
0
**
Week 1
Week 3
Age
C
LS mean glycogen
0.6
0.4
0.2
0.0
**
Week 1
Week 3
Age
D
LS mean triacylglycerol
0.4
0.3
0.2
0.1
0.0
**
****
Week 1
Week 3
Age

Figure 5

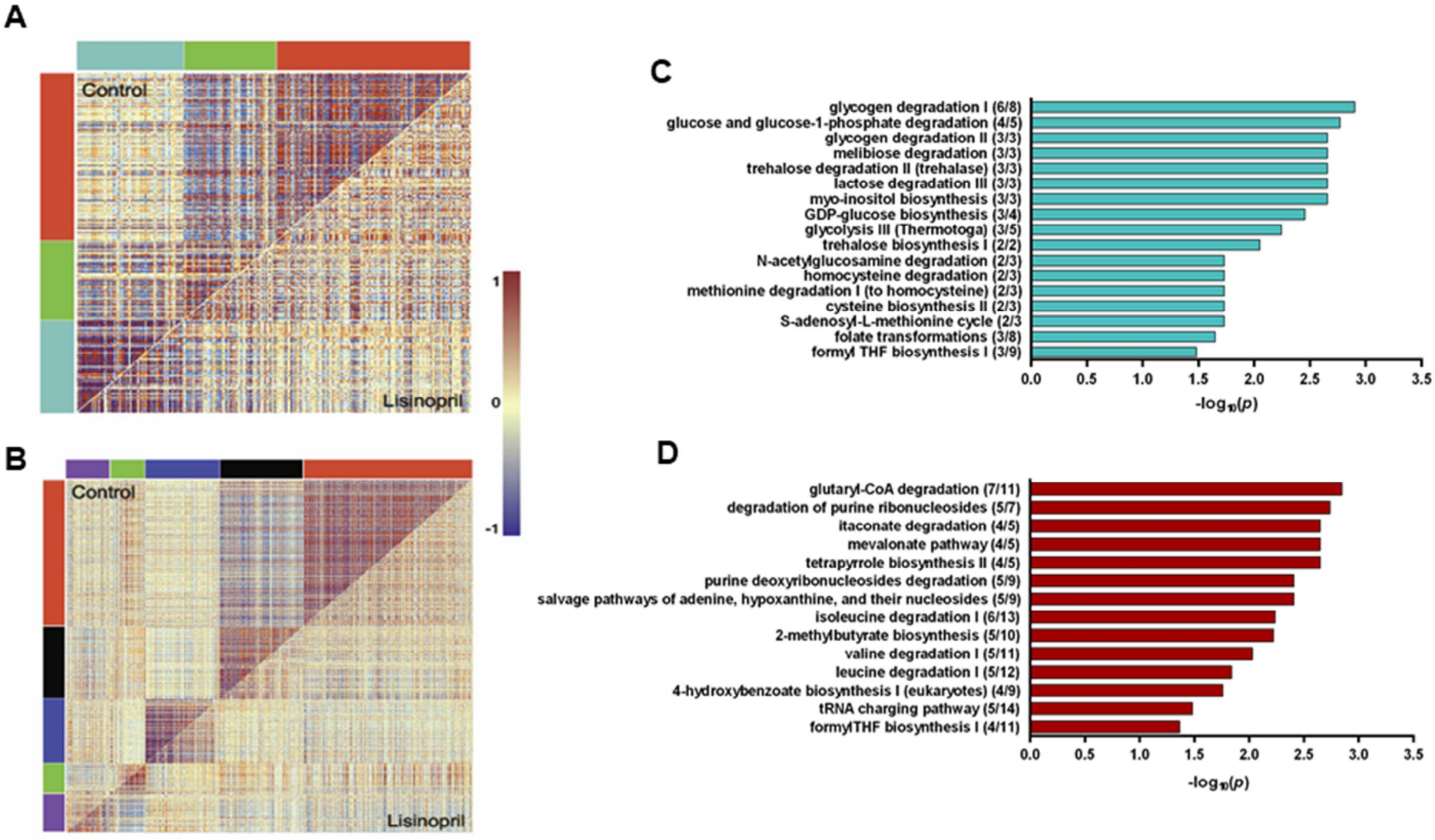
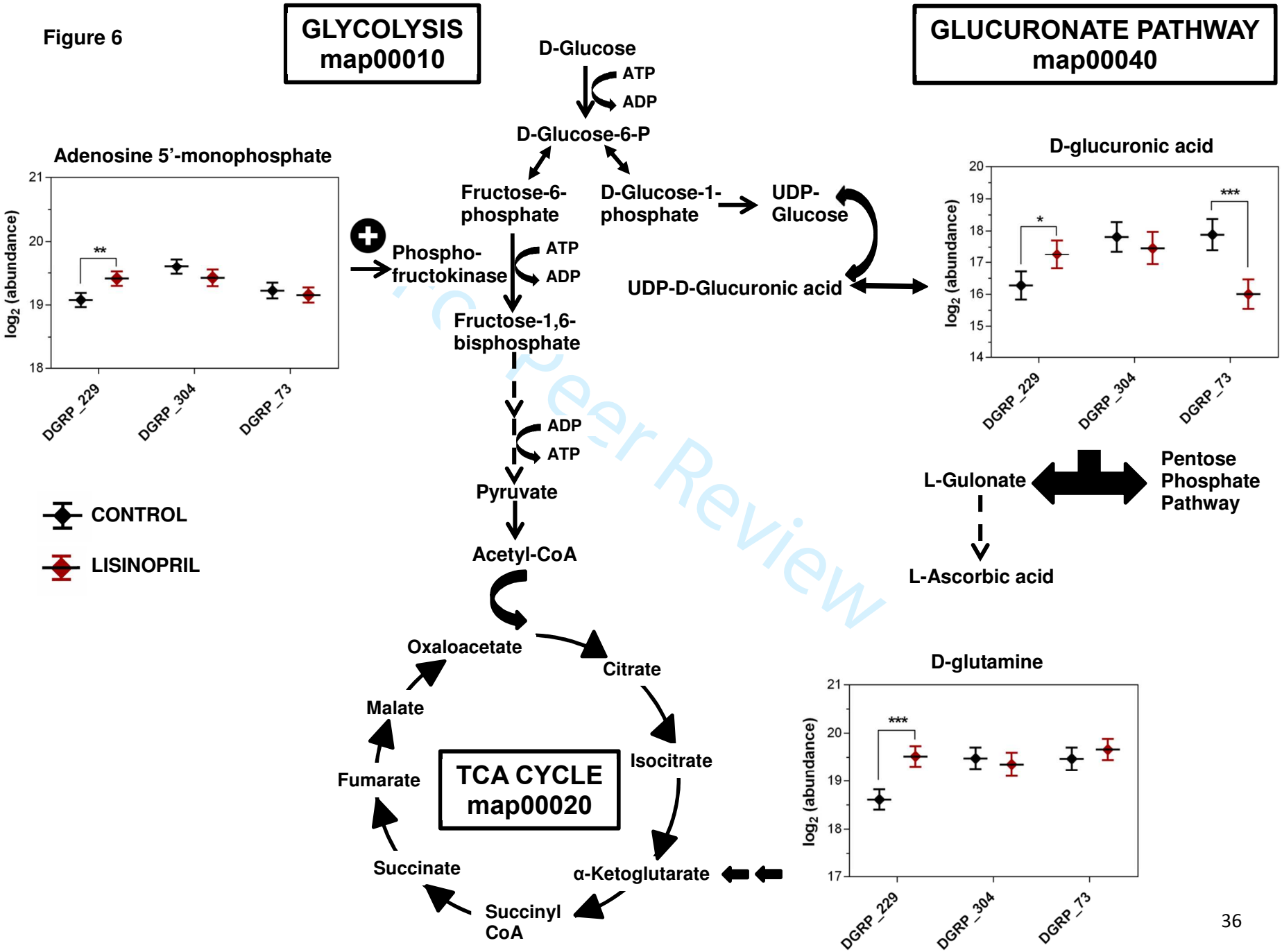

A
Control
Lisinopril
1
0
-1
B
Control
Lisinopril
C
glycogen degradation I (6/8)
glucose and glucose-1-phosphate degradation (4/5)
glycogen degradation II (3/3)
melibiose degradation (3/3)
trehalose degradation II (trehalase) (3/3)
lactose degradation III (3/3)
myo-inositol biosynthesis (3/3)
GDP-glucose biosynthesis (3/4)
glycolysis III (Thermotoga) (3/5)
trehalose biosynthesis I (2/2)
N-acetylglucosamine degradation (2/3)
homocysteine degradation (2/3)
methionine degradation I (to homocysteine) (2/3)
cysteine biosynthesis II (2/3)
S-adenosyl-L-methionine cycle (2/3
folate transformations (3/8)
formyl THF biosynthesis I (3/9)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
-log10(p)
D
glutaryl-CoA degradation (7/11)
degradation of purine ribonucleosides (5/7)
itaconate degradation (4/5)
mevalonate pathway (4/5)
tetrapyrrole biosynthesis II (4/5)
purine deoxyribonucleosides degradation (5/9)
salvage pathways of adenine, hypoxanthine, and their nucleosides (5/9)
isoleucine degradation I (6/13)
2-methylbutyrate biosynthesis (5/10)
valine degradation I (5/11)
leucine degradation I (5/12)
4-hydroxybenzoate biosynthesis I (eukaryotes) (4/9)
tRNA charging pathway (5/14)
formylTHF biosynthesis I (4/11)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
-log10(p)

Figure 6



Supplementary Information

Genotype- and age-specific effects of Lisinopril on mitochondrial biogenesis and function, reactive oxygen species levels, and metabolomic profiles in *Drosophila melanogaster*

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Supplemental Methods	Liquid chromatograph-tandem mass spectrometry-based detection of Lisinopril, mitochondrial complex activities assays, and global metabolomic profiling
Table S1.	Mixed model ANOVA/ANCOVAs of thoracic mitochondria-related phenotypes
Table S2.	Mixed model ANOVA of thoracic H_2O_2 levels
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Table S4.	List of metabolites detected by high-resolution LC-MS in the thoraces of three Drosophila Genome Reference Panel strains at one and three weeks of age
Table S5.	Untargeted metabolomics analysis results
Table S6.	*Mummichog* pathway enrichment analysis for modules of differentially co-regulated metabolite features between Lisinopril treated and control flies
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Supplemental Methods

Liquid chromatograph-tandem mass spectrometry (LC-MS/MS)-based detection of Lisinopril

Ten pooled flies fed 1mM Lisinopril and controls were homogenized 1:20 with 5 mM ammonium acetate buffer using a pellet pestle. Calibration standards, blanks and Quality Controls were prepared by spiking naïve fly homogenate (100 µL) with the appropriate amount of Lisinopril to achieve concentrations in the tissue homogenate ranging from 50-10,000 ng/mL. Standards, blanks, quality controls (QCs), and samples were spiked with internal standard (10 µL of a 100 ng/mL Enalaprat) and proteins were precipitated by the addition of 0.5 mL of 90:10 methanol:acetone solution followed by vortex mixing. After centrifugation for 5 minutes at 21,000 x g, the supernatant was transferred to culture tubes and evaporated under a stream of dry nitrogen at 50℃. The residue was dissolved in distilled water, vortexed, transferred to a limited volume autosampler vial, and analyzed in positive ion mode LC-MS/MS. The LC-MS/MS system consisted of Shimadzu system (Columbia MD) equipped with LC20-AD dual HLPC pumps, an SIL20-AC HT autosampler, and a DGU-20A2 in-line degasser. Detection was performed using an Applied BioSystems 4000 QTRAP (Applied Biosystems, Foster City, CA) triple quadrupole mass spectrometer operated in the positive ion mode utilizing electrospray ionization. Mass calibration, data acquisition and quantitation were performed using Applied Biosystems Analyst 1.6.2 software (Applied Biosystems, Foster City, CA). Separation of the Lisinopril and the internal standard from the homogenate matrix was achieved using a Phenomenex Luna C18, 100 X 2 mm 5 µm particle column kept at 40℃. The mobile phase was delivered at a flow rate of 400 µL/min using a gradient elution profile consisting of distilled water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The elution gradient began at of 5% of 0.1% formic acid and after 1.5 minutes increased linearly to 80% over 4 minutes. After a 0.5-minute hold, the gradient was changed in a step fashion back to 5% and re-equilibrated for 1.5 minutes. The analyte and internal standard were detected using multiple reaction monitoring using the following ion transitions: Lisinopril (m/z 406.4→84), Enalaprat (m/z 349.2→ 206.1).

Differences in Lisinopril concentration among strains were analyzed in ANOVA. We used the Tukey test for *post hoc* pairwise comparisons of differences among genotypes.

Mitochondrial complex activities assays

Complex I activity was immediately measured on a DU800 spectrophotometer using 2,6-dichloroindophenol (DCIP) as the terminal electron acceptor at 600 nm with the oxidation of NADH reducing artificial substrates Coenzyme Q10 that then reduces DCIP. The reduction of DCIP is mostly dependent on complex I activity and has a very high rotenone sensitive activity (Janssen *et al.* 2007). Complex IV activity was measured by the oxidation of cytochrome c at 550 nm (Ragan *et al.* 1987). Data are represented as the pseudo first order rate constant (k) divided by protein concentration. Complex V activity, measured in the direction of ATP hydrolysis was assayed by the continuous spectrophotometric monitoring of the oxidation of NADH (e340 = 6180 M-1cm-1) in an enzyme-linked ATP regenerating assay using ATP, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase to determine the ATPase activity (NADH loss) in nmol/min/mg protein (Feniouk *et al.* 2007).

Global metabolomic profiling

Metabolite detection

Each *Drosophila* sample was weighed and then homogenized in 200 µL water with 10% PBS (1x) in an Eppendorf tube while immersed in an ice bath. Methanol (800 µL) was then added, followed by vortexing for 2 min to precipitate proteins and incubation at -20 ℃ for 30 min. Samples were sonicated in an ice bath for 10 min and then centrifuged at 14000 rpm for 5 min at 4 ℃. From each tube, 900 µL supernatant was transferred to a new Eppendorf tube for drying under vacuum at 30 ℃ (~3 hrs). The completely dried samples were reconstituted in 100 µL 40% water/60% ACN for MS analysis. A pooled QC sample was then made by combining small aliquots (~5 µl) from each reconstituted sample. This pooled QC was analyzed once for every 10 study samples to serve as a technical replicate throughout the data set to assess process reproducibility and allow for data normalization to account for any instrument drift.

LC-MS analysis was performed using an LC-QTOF-MS system (Agilent Technologies, Santa Clara, CA) consisting of an Agilent 1200 SL liquid chromatography system coupled online with an Agilent 6520 time-of-flight mass spectrometer. A 5 µL aliquot of reconstituted sample was injected onto a 2.1×150 mm Waters BEH-Amide 2.5 µm particle column at 35 ℃. The metabolites were gradient-eluted at 0.3 mL/min using mobile phase A, 5 mM ammonium formate and 0.0125% formic acid in 97% water/3% ACN, and mobile phase B, 5 mM ammonium formate and 0.0125% formic acid in 3% water/97% ACN (98% B for 1 min, 98% to 77% B in 6.5 min, 77% to 39% B in 4.5 min and 39% B for 7 min). The MS interface capillary was maintained at 325 ℃ with a nebulizing gas pressure of 45 psig, and a drying gas flow of 9 L/min. The capillary voltage for positive ion injection was 3.5 kV. Agilent MassHunter Workstation Data Acquisition software B.02.01 (B2116.30) was used to acquire all data from 60 to 1000 m/Z using centroid mode with a threshold of 200 or 0.01%.

Data analysis

We performed a median normalization where we adjusted the data so all samples would have the same median value of the metabolite abundance post log_2 transformation. We then selected metabolites with < 5% missingness and imputed the remaining missing data using the K-nearest neighbor (KNN) algorithm (Troyanskaya *et al.* 2001). Briefly, for each metabolite with missing values, we found the K nearest neighbors (where K=10) using a Euclidean distance, confined to the columns (samples) for which that metabolite is not missing. For every metabolite, the missing values were then replaced with the average of the non-missing values of its neighbors. We then fitted a weighted linear model to the data using the Bioconductor limma package (Ritchie *et al.* 2015), with coefficients for treatment, genotype, age, and interaction terms. The limma package uses empirical Bayes moderated statistics, which improves power by 'borrowing strength' between metabolites in order to moderate the residual variance (Smyth 2004). The sample-specific weights allowed us to up or down-weight individual samples (Ritchie et al. 2006). Metabolite changes in response to Lisinopril were considered significant with a false discovery rate of 10% to account for multiple testing. Since there are three genotypes, we

performed a moderated F-test first in those cases where there are more than one interaction terms in the model.

To identify modules of metabolites differentially co-expressed between Lisinopril and control treatments, we applied the differential co-expression method, DiffCoEx (Tesson *et al.* 2010), which takes advantage of methods from the Weighted Gene Network Correlation Analysis (WGCNA) package in R (Langfelder & Horvath 2008). Briefly, WGCNA generates a correlation matrix of all metabolic features across all observations from the dataset and applies a clustering algorithm to identify clusters (or modules) of related features. DiffCoEx takes these identified modules and evaluates the difference in their abundance levels across two environments (in our case, Lisinopril *vs* control) to identify modules that are differentially regulated across these two environments. Different input parameters can be adjusted when using DiffCoEx. In our analysis, we set the scaling coefficient $\beta = 7$ and set minimum module size to 20 features. DiffCoEx identifies modules of features that show similar changes between treatment and control. We used the software package *mummichog* (Li *et al.* 2013) to assign pathway Ids and to test for functional enrichment within each set of metabolite features associated with a specific module.

Table S1. Mixed model ANOVA/ANCOVAs of thoracic mitochondria-related phenotypes. Age is a fixed effect, Genotype is a random effect, CS (Citrate Synthase activity) is a covariate; df: degrees of freedom; SS: Type III Sums of Squares, MS: Mean square

Phenotype	Source of Variation	df	SS	MS	F-value	*P*-value
State 3 respiration rate	CS	1	12865	12865	2.47	0.1233
	Genotype	3	129775	43258	0.61	0.6527
	Age	1	156048	156048	2.74	0.1916
	Genotype×Age	3	232937	77646	14.92	<.0001
	Error	43	223825	5205		
State 2 respiration rate	CS	1	2176	2176	21.65	<.0001
	Genotype	3	2261	754	1.07	0.4763
	Age	1	767	767	1.33	0.3242
	Genotype×Age	3	2296	765	7.61	0.0003
	Error	43	4322	101		
State 4o respiration rate	CS	1	631	631	4.50	0.0397
	Genotype	3	3222	1074	1.15	0.4535
	Age	1	466	466	0.61	0.4865
	Genotype×Age	3	3045	1015	7.23	0.0005
	Error	43	6035	140		
mtDNA/nDNA	Genotype	3	0.153	0.051	174.01	0.0007
	Age	1	0.002	0.002	3.24	0.1974
	GenotypexAge	3	0.001	0.0003	0.17	0.9132
	Error	35	0.059	0.002		

Table S2. Mixed model ANOVA of thoracic H_2O_2 levels. Age is a fixed effect, Genotype is a random effect; df: degrees of freedom; SS: Type III Sums of Squares, MS: Mean square;

Source of Variation	df	SS	MS	F-value	*P*-value
Genotype	3	56.01	18.67	14.15	0.0282
Age	1	13.54	13.54	10.27	0.0478
GenotypexAge	3	3.96	1.32	1.06	0.3719
Error	68	84.66	1.24		

Table S3. Mixed model ANOVA/ANCOVAs of thoracic mitochondria-related phenotypes, H_2O_2 levels, and triacylglycerol and glycogen content in Lisinopril treated and control flies. Treatment and Age are fixed effects, Genotype is a random effect; CS (citrate cynthase activity) and PRO (total protein levels) are covariates; df: degrees of freedom; SS: Type III Sums of Squares, MS: Mean square;

Phenotype	Source of Variation	df	SS	MS	F-value	*P*-value
State 3 respiration rate	CS	1	7691	7691	1.34	0.2511
	Treatment	1	11114	11114	1.22	0.6451
	Genotype	3	288102	96034	1.14	0.4619
	Age	1	169794	169794	2.85	0.1989
	Treatment*Genotype	3	45907	15302	0.98	0.5054
	Treatment*Age	1	9387	9387	0.60	0.4934
	Genotype*Age	3	275913	91971	6.49	0.0670
	Treatment*Genotype*Age	3	46611	15537	2.70	0.0509
	Error	84	483703	5758		
mtDNA/nDNA	Treatment	1	0.007	0.007	3.14	0.8802
	Genotype	3	0.302	0.100	74.21	0.8705
	Age	1	0.027	0.027	4.74	0.5695
	Treatment*Genotype	3	0.011	0.004	0.39	0.7686
	Treatment*Age	1	0.008	0.008	0.83	0.4294
	Genotype*Age	3	0.022	0.007	0.83	0.4289
	Treatment*Genotype*Age	3	0.029	0.010	4.88	0.0040
	Error	67	0.134	0.002		
H_2O_2 levels	Treatment	1	4.27	4.27	0.77	0.5962
	Genotype	3	70.42	23.47	6.28	0.2602
	Age	1	62.24	62.24	7.88	0.1856
	Treatment*Genotype	3	6.92	2.31	0.71	0.6081
	Treatment*Age	1	6.50	6.50	2.02	0.2492
	Genotype*Age	3	14.06	4.69	1.44	0.3864
	Treatment*Genotype*Age	3	9.78	3.26	3.06	0.0300
	Error	147	156.36	1.06		

Phenotype	Source of Variation	df	SS	MS	F-value	*P*-value
Triacylglycerol	PRO	1	0.001	0.001	0.95	0.3308
	Treatment	1	0.03	0.03	0.75	0.5137
	Genotype	3	0.02	0.01	0.21	0.8815
	Age	1	0.04	0.04	0.74	0.4918
	Treatment*Genotype	3	0.03	0.01	2.70	0.2246
	Treatment*Age	1	0.03	0.03	8.64	0.0536
	Genotype*Age	3	0.05	0.02	4.37	0.1217
	Treatment*Genotype*Age	3	0.01	0.004	3.84	0.0111
	Error	136	0.13	0.001		
Glycogen	PRO	1	0.005	0.005	0.80	0.3743
	Treatment	1	0.02	0.02	0.58	0.5723
	Genotype	3	0.31	0.10	2.99	0.2753
	Age	1	0.001	0.001	0.06	0.8613
	Treatment*Genotype	3	0.10	0.03	1.60	0.3528
	Treatment*Age	1	0.02	002	1.09	0.3618
	Genotype*Age	3	0.07	0.02	1.09	0.4736
	Treatment*Genotype*Age	3	0.07	0.02	3.55	0.0173
	Error	98	0.60	0.01		

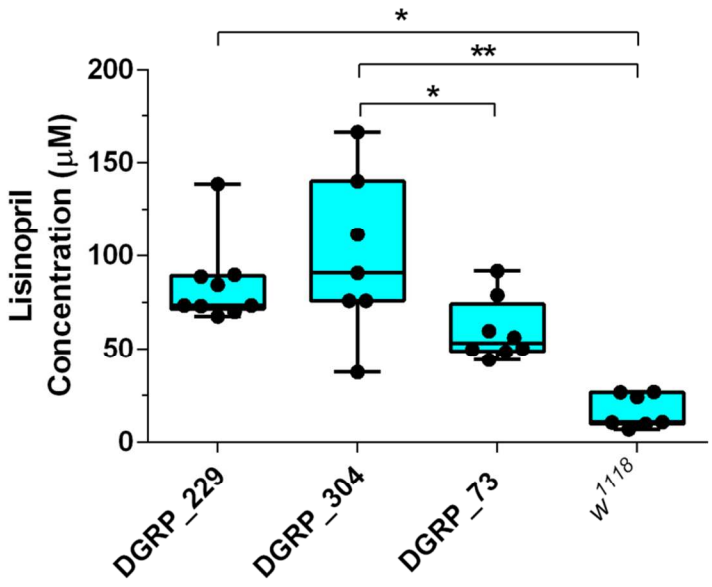

Figure S1. Lisinopril concentration in whole-body homogenates. Box and whiskers plots denote individual data points separated by a line representing the group median. Each individual value is plotted as a dot superimposed on the boxplots. *p<0.05 and **p<0.01. Error bars represent standard errors.

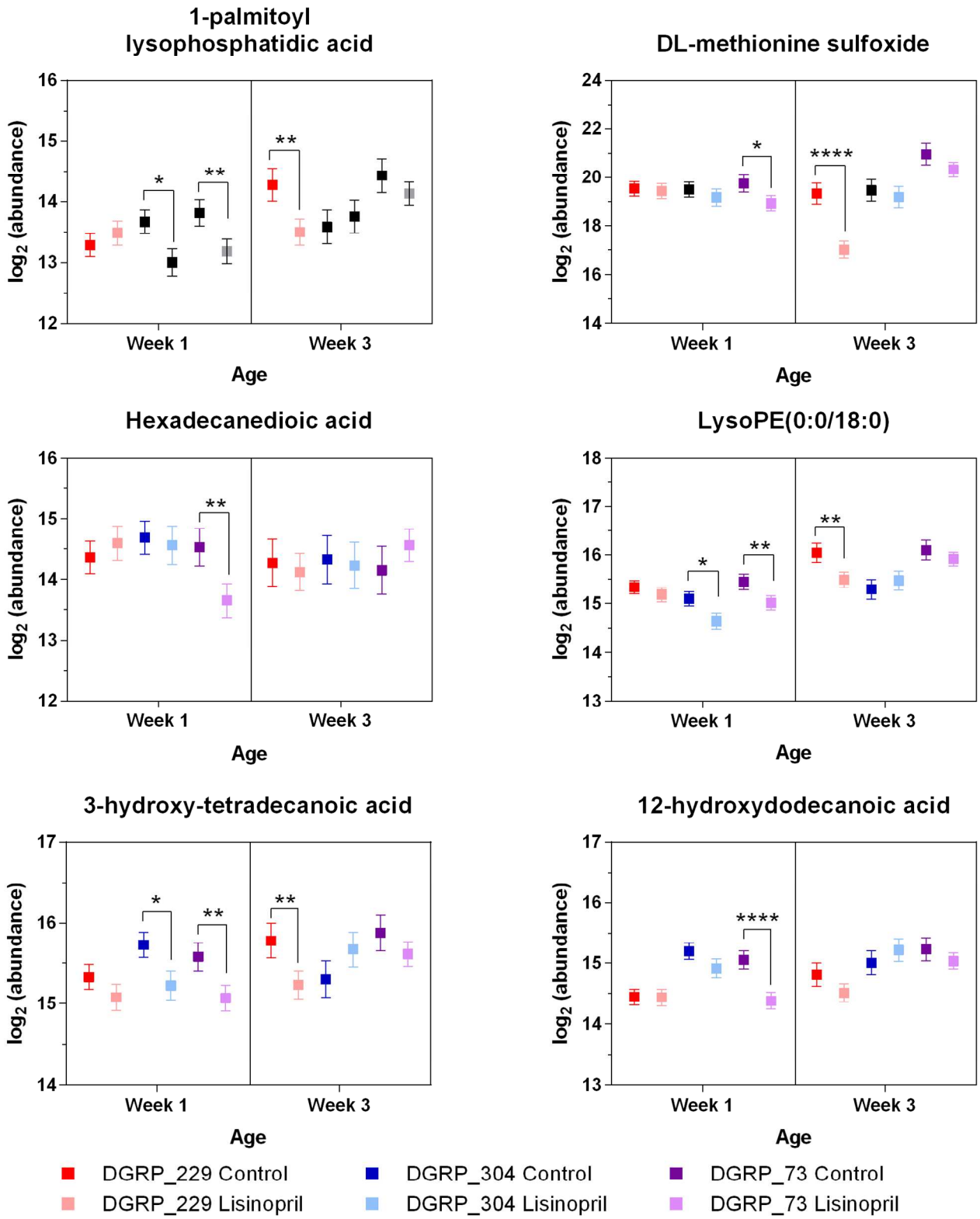

Figure S2. Metabolites with genotype- and age-specific changes in response to Lisinopril. Data reported on the plots denote the mean *log2* abundance of three replicate samples for each treatment, genotype, and age group. *p<0.05, **p<0.01, and ****p<0.0001 after Benjamini and Hochberg's adjustment for multiple comparisons. Error bars represent the 95% confidence interval.

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ORIGINAL ARTICLE

Reduction of *Syndecan* Transcript Levels in the Insulin-Producing Cells Affects Glucose Homeostasis in Adult *Drosophila melanogaster*

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Signaling by direct cell–matrix interactions has been shown to impact the transcription, secretion, and storage of insulin in mammalian β cells. However, more research is still needed in this area. Syndecans are transmembrane heparan sulfate proteoglycans that function independently and in synergy with integrin-mediated signaling to mediate cell adhesion to the extracellular matrix. In this study, we used the model organism *Drosophila melanogaster* to determine whether knockdown of the *Syndecan* (*Sdc*) gene expression specifically in the insulin-producing cells (IPCs) might affect insulin-like peptide (ILP) production and secretion. IPCs of adult flies produce three ILPs (ILP2, ILP3, and ILP5), which have significant homology to mammalian insulin. We report that flies with reduced *Sdc* expression in the IPCs did not show any difference in the expression of *ilp* genes compared to controls. However, they had significantly reduced levels of the circulating ILP2 protein, higher circulating carbohydrates, and were less glucose tolerant than control flies. Finally, we found that IPCs-specific *Sdc* knockdown led to reduced levels of head *Glucose transporter1* gene expression, extracellular signal-regulated kinase phosphorylation, and reactive oxygen species. Taken together, our findings suggest a cell autonomous role for Sdc in insulin release in *D. melanogaster*.

Keywords: syndecan, glucose-induced insulin secretion, ERK, AKT

Introduction

Syndecans are cell-surface transmembrane proteins that have been conserved throughout metazoan evolution and are present on adherent cells (Chakravarti and Adams, 2006). They belong to the family of heparan sulfate proteoglycans that also include glycosylphosphatidylinositol-anchored glypicans and secreted proteoglycans found in the extracellular matrix (ECM) (Morgan *et al.*, 2007; Xian *et al.*, 2010). Although invertebrates have only one *Syndecan* (*Sdc*) gene and protein, there are four genes (*SDC1*, *SDC2*, *SDC3*, and *SDC4*) and corresponding proteins in vertebrates (Chakravarti and Adams, 2006). Structurally, however, all syndecans are composed by a core protein with extracellular attachment sites for glycosaminoglycan chains that mediate interactions with a wide array of ligands, including ECM components and soluble growth factors. The extracellular domain extends into a transmembrane domain and a highly conserved short cytoplasmic tail (Morgan *et al.*, 2007; Xian *et al.*, 2010). The latter binds to cytoskeleton proteins and allows syndecans to function independently and in synergy with integrin-mediated signaling to mediate cell adhesion to the ECM (Morgan *et al.*, 2007; Bellin *et al.*, 2009; Xian *et al.*, 2010; Couchman *et al.*, 2015). Engagement of ECM proteins to integrins and/or syndecans triggers mechanical and biochemical alterations, such as activation of the focal adhesion kinase, phosphoinositide-3-kinase/Akt (PI3K/Akt), and Ras/extracellular signal-regulated kinase (ERK) cascade, which convey a signal from the cell membrane through the cytoskeleton to the nucleus (Bellin *et al.*, 2009; Provenzano and Keely, 2011). This leads to chromatin remodeling and gene expression changes that, in turn, reorganize and remodel the ECM compartment (Nelson and Bissell, 2006). Thus, a state of ''dynamic reciprocity'' exists between cells and their ECM, which plays a fundamental role in regulating tissue structure and function (Nelson and Bissell, 2006). Several studies in humans and rodents strongly suggest that the protein composition and dynamics of the ECM in several metabolic tissues play a critical role in the link between obesity and metabolic complications

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(Williams *et al.*, 2015). However, we still know little about the impact of alterations in expression of mediators of cell–ECM interaction, such as syndecans, on the maintenance of energy balance and homeostasis.

The fly *Drosophila melanogaster* is a powerful model for studying human metabolic regulation (Owusu-Ansah and Perrimon, 2014). The power of *Drosophila* lies not only in its well-characterized biology, low cost, and excellent genetic and genomic resources, but also in the fact that nutrient-sensing signaling pathways and regulatory mechanisms involved in energy homeostasis are highly conserved (Leopold and Perrimon, 2007). For instance, as in mammals, circulating sugar levels are regulated by insulin-like peptides (ILPs) that are secreted into the circulating hemolymph (Leopold and Perrimon, 2007). The *Drosophila* genome contains eight *ilp* genes: *ilp1* through *-8*. In adult flies, *ilp2*, *ilp3*, and *ilp5*, which show significant homology to mouse and human insulin, are expressed in a set of neurosecretory cells of the *pars intercerebralis* in the brain known as the insulin-producing cells (IPCs) (Nassel *et al.*, 2013). Among the *ilp* genes, *ilp2* is the most expressed in the IPCs, the most abundant ILP in circulation, and the closest homolog of human insulin (Nassel *et al.*, 2013). The IPCs of adult flies display several commonalities with the human β cells with respect to insulin release, relying on membrane depolarization and influx of calcium in response to glucose for release of the IPCs-derived ILPs (Kreneisz *et al.*, 2010; Barry and Thummel, 2016). Partial genetic deletion of IPCs in the adult fly produces phenotypes similar to those observed in human diabetic patients (Haselton and Fridell, 2010). These include increased circulating carbohydrate (Broughton *et al.*, 2008; Haselton *et al.*, 2010) and impaired ability to clear carbohydrate from circulation following food intake (Haselton and Fridell, 2010). Homologs of members of the insulin-signaling pathway are also found in *Drosophila* and function similar to their mammalian cognates (Garofalo, 2002; Teleman, 2009).

We previously showed that adult *Sdc* mutant flies displayed reduced whole-body energy metabolism compared to controls (De Luca *et al.*, 2010). Following up on this observation we reported that knocking down *Sdc* specifically in the fat body (the insect equivalent of mammalian adipose tissue and liver) perturbed energy balance and metabolism (Eveland *et al.*, 2016). Compared to controls, fat body-specific *Sdc* knockdown flies also exhibited a significant decrease in phosphorylation levels of PI3K/Akt and ERK (Eveland *et al.*, 2016), suggesting that changes in cell survival signaling pathways may mediate the effect of reduced fat body Sdc on metabolism (Kuznetsov *et al.*, 2004). One additional finding in our initial study was that hypomorphic *Sdc* mutant flies had lower expression of head *ilp* genes than controls (De Luca *et al.*, 2010). In mammals, the interaction between ECM components and their cell surface receptors has been shown to impact the transcription, secretion, and storage of insulin (Kaido *et al.*, 2006). We, therefore, reasoned that deficiency of *Sdc* in the IPCs might also affect *Drosophila* ILP2 production. To test our idea, we used the GAL4/UAS-RNA interference (RNAi) approach (Duffy, 2002) to knockdown the expression of the *Sdc* gene specifically in the IPCs. The results of the present study provide initial evidence of a role for Sdc in the glucose-induced release of circulating ILP2 in adult *D. melanogaster*.

Materials and Methods

Drosophila *strains*

The UAS-*Sdc*-RNAi line (Dietzl *et al.*, 2007) was obtained from the Vienna Stock Center (Stock ID: v13322) and its knockdown efficiency was previously reported (Eveland *et al.*, 2016). The w^{1118} wild-type line, from which the *UAS-Sdc*-RNAi line was originally generated (http://flystocks.bio.indiana.edu/Browse/VDRCtb.htm), was obtained from the Bloomington Stock Center and used as control line. The *ilp2-Gal4* line was a generous gift from Dr. Eric Rulifson (University of California, San Francisco). The w^{1118}*; ilp2-Gal4/+; UAS- Sdc*-RNAi/+ (hereinafter referred to as ilp2>Sdc-RNAi) and w^{1118}*; ilp2-Gal4/+; +/+* (hereinafter referred to as ilp2>w1118) genotypes originated from a cross between *ilp2-Gal4* males and *UAS-Sdc-IR* or w^{1118} female flies. All flies were reared in temperature-controlled incubators at 25°C and 60–75% relative humidity on a standard cornmeal, molasses, and yeast medium with 12 h light-dark cycles. Newly enclosed male flies were collected as virgins and aged 4–6 days before being assessed for the phenotypic assays described below.

Quantitative polymerase chain reaction

Flies were snap-frozen in liquid nitrogen between 9:00 and 10:00 am on collection days and stored at −80°C. Total RNA was extracted from fly heads using the Qiagen RNeasy Mini column filtration kit (Qiagen, Valencia, CA) following the manufacturer's protocols. cDNA was then made using the First Strand Synthesis kit (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reactions (qPCRs) were performed using the protocol described in Jumbo-Lucioni *et al.* (2012).

Enzyme-linked immunosorbent assay

The ILP2 antibody was a generous gift from Dr. Ernst Hafen (Institute of Molecular Systems Biology, ETH Zurich). Hemolymph samples were collected using the protocol described in Park *et al.* (2014). Briefly, Zymo-spin IIIC columns (Zymo Research Corporation, Irvine, CA) were filled with 60 flies after removing the DNA binding matrix and centrifuged twice at 9000 *g* for 5 min at 4°C to collect hemolymph. Circulating ILP2 levels were measured by enzyme-linked immunosorbent assay (ELISA) using the protocol described in Bai *et al.* (2012).

Oral-glucose tolerance test

The oral-glucose tolerance test (OGTT) assay was adapted from Barry and Thummel (2016) and Haselton and Fridell (2010). Six replicates per time point and genotype, each containing five flies, were fasted overnight on a 1% agar solution. After fasting, one set of six replicates per genotype was frozen (''fasted'' time). Replicate groups were refed in vials containing 10% glucose/1% agar medium for 2 h. While one set of replicates was frozen (''fed'' time), the remaining replicate groups were transferred back to the fasting medium and frozen after 2 or 4 h postprandial (''2 or 4 h re-fast'' times). Frozen flies were then homogenized in 100 μL phosphate-buffered saline and glucose was measured using the protocol described in Eveland *et al.* (2016).

Metabolic and feeding assays

Total triacylglycerol, glycogen, and protein levels were measured using protocols described in Eveland *et al.* (2016) and Jumbo-Lucioni *et al.* (2010). Circulating trehalose was measured in hemolymph using the method described in Eveland *et al.* (2016) and Park *et al.* (2014).

Metabolic rate was measured as CO_2 production using a flow-through respirometry system (Qubit System Research, Kingston, Ontario, Canada) and the approach was described in Jumbo-Lucioni *et al.* (2010).

Food intake was assessed using the Capillary Feeder assay described in Eveland *et al.* (2016) and Ja *et al.* (2007).

Starvation resistance assay

Survival under starvation conditions was assessed as described in De Luca *et al.* (2010).

Western blotting

Fly heads or bodies were snap-frozen and stored at –20°C. Frozen tissues were then homogenized in standard lysis buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Thermo Scientific, Rockford), blocked in 5% bovine serum albumin, and incubated with 1:1000 rabbit β-Actin (# 4967; Cell Signaling, Danvers, MA); 1:1000 rabbit anti-Phospho-*Drosophila* Akt (Ser505) (#4054; Cell Signaling) and rabbit anti-Akt (#9272; Cell Signaling); 1:1000 rabbit anti- Phospho-ERK/MAPK (#4377; Cell Signaling) and rabbit anti-ERK/MAPK (#4695; Cell Signaling). The primary antibodies were incubated overnight at 4°C. The secondary antibody was incubated with 1:5000 horseradish peroxide-conjugated goat anti-rabbit or goat anti-mouse for 1 h at room temperature and the signal developed using an enhanced chemiluminescence detection kit (Merk Millipore, Billerica, MA).

H_2O_2 measurements

Ten brains per genotype were dissected from live flies in 20 mM N-ethyl maleimide, which is a cell permeable alkylating agent that prevents air-induced oxidation during fly dissection (Albrecht *et al.*, 2011). Brain H_2O_2 levels were quantified using the Fluorimetric Hydrogen Peroxide Assay Kit (#MAK165-1KT; Sigma-Aldrich) according to the manufacturer's instructions. Fluorescence ($\lambda_{ex} = 540/\lambda_{em} = 590$ nm) was measured with a BioTek microplate reader (BioTek Instruments, Winooski, VT).

Statistical analyses

Cox regression was used to compare survival of ilp2>Sdc-RNAi and ilp2>w1118 flies under starvation conditions using SAS 9.4 software (SAS Institute, Cary, NC). One-way analysis of covariance (with body weight used as covariate) was used to assess differences between experimental and control groups in resting metabolic rate. Data from the other experimental assays were analyzed by two-tailed Student's *t*-test.

Results

Knockdown of Sdc in IPCs perturbs circulating ILP2 levels and glucose metabolism

We first analyzed the effects of *Sdc* knockdown in the IPCs on the expression of *ilp2*, *ilp3*, and *ilp5* genes. Contrary to our prediction, we found no significant differences in transcription levels of any of the *ilp* genes between ilp2>Sdc-RNAi and ilp2>w1118 flies (Fig. 1A). However, quantification of circulating ILP2 in extracted hemolymph revealed that IPCs-specific *Sdc* knockdown flies had significantly less (45%) circulating ILP2 levels than controls (Fig. 1B).

Genetic manipulation of ILP2 production alters the levels of trehalose (the major circulating carbohydrate in the fly) in the hemolymph (Grönke *et al.*, 2010; Haselton and Fridell, 2010). Therefore, we next measured circulating trehalose in our experimental and control flies. As expected, there was a significant increase (52%) in hemolymph trehalose in flies with *Sdc* knocked down in the IPCs relative to controls (Fig. 2A). Previous studies also reported that IPCs-ablated flies had higher levels of stored metabolites (glycogen and triacylglycerol) and were more resistant to starvation than control flies, likely due to the increased levels of nutrient reserves (Broughton *et al.*, 2005). Consistent with these observations, we found that not only body glycogen levels were significantly higher (66%) in ilp2>Sdc-RNAi flies (Fig. 2B), but they also lived significantly longer than ilp2>w1118 flies under starvation conditions (Fig. 2C). In contrast, no changes in body triacylglycerol stores between groups were observed (Fig. 2B).

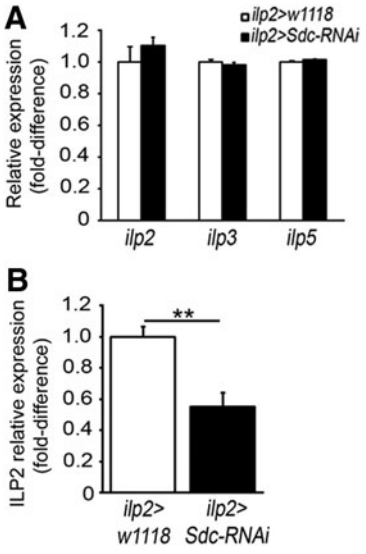

FIG. 1. IPCs-specific *Sdc* knockdown leads to significantly decreased circulating ILP2 protein levels. **(A, B)** Despite no decrease in *ilps* mRNA levels **(A)**, circulating ILP2 levels were significantly lower in IPC-specific *Sdc* knockdown (ilp2<Sdc-RNAi) flies compared to controls (ilp2<w1118) **(B)**. Gene expression levels were measured by qPCR using mRNA extracted from heads of males ($n = 6$). All genes were normalized to *rp49*. Circulating ILP2 levels were measured by ELISA and values represent the means of four independent replicates. $^{**}p < 0.01$ relative to control. Error bars represent SE. ELISA, enzyme-linked immunosorbent assay; ILP, insulin-like peptide; IPC, insulin-producing cell; qPCR, quantitative polymerase chain reaction; SE, standard error.

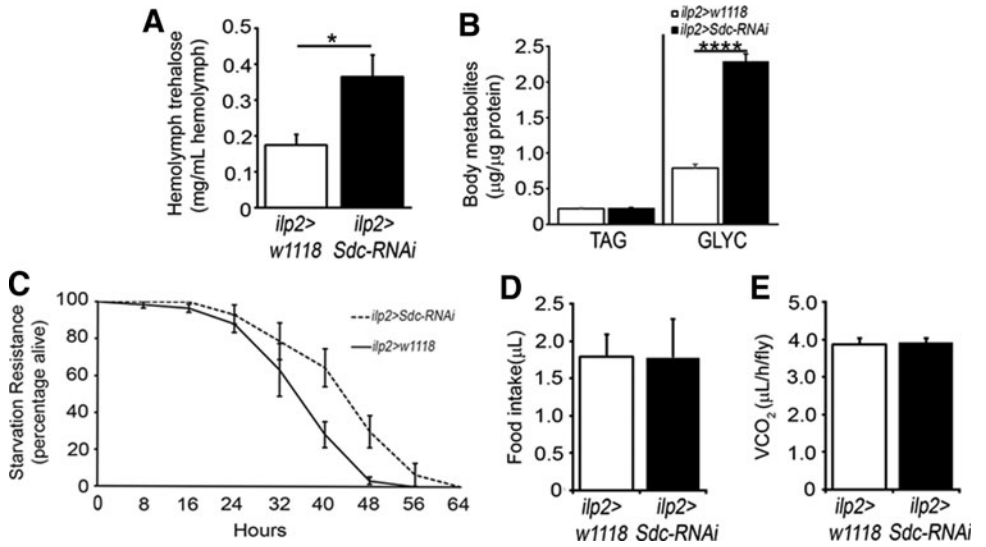

FIG. 2. IPCs-specific *Sdc* knockdown increases circulating trehalose and stored glycogen, and provides survival advantage to starvation independent of changes on energy balance. **(A)** Values represent the mean of circulating trehalose levels in IPCs-specific *Sdc* knockdown (ilp2>Sdc-RNAi) and control (ilp2>w1118) flies ($n \geq 7$). **(B)** Levels of triacylglycerol (TAG, *left*) and glycogen (GLYC, *right*) in homogenates of decapitated flies ($n = 10$ independent replicates). Measurements were normalized to total protein levels. **(C)** Survival curves under water-only starvation conditions. Starvation survival times were significantly longer in ilp2>Sdc-RNAi males compared to controls [log-rank test, chi square statistic (χ^2) = 16.3, $p < 0.0001$]. **(D)** Values represent the average amount of food ingested by five housed flies from 10 independent replicates. **(E)** Values represent the least-square means of whole-body CO_2 production (VCO_2), an index of resting metabolic rate, adjusted for live body weight ($n = 10$ independent replicates). $^*p < 0.05$ and $^{****}p < 0.0001$ relative to control. Error bars represent SE.

To confirm that the effects on glucose metabolism were due to differences in the levels of circulating ILP2 between ilp2>Sdc-RNAi and ilp2>w1118 flies rather than alterations in energy balance, we measured food intake and resting metabolic rate. We found no differences in either food intake (Fig. 2D) or in metabolic activity (Fig. 2E) between genotypes.

Knockdown of Sdc in IPCs leads to reduced glucose tolerance

Given that ilp2>Sdc-RNAi flies had reduced circulating ILP2 levels, we performed an OGTT assay to assess whether they would take longer than ilp2>w1118 flies to restore their glucose levels to normal following ingestion of a large amount of glucose. As shown in Figure 3A, whereas there was no significant distinction in body glucose levels between ilp2>Sdc-RNAi and control flies after an overnight fast (''fasted''), we found that ilp2>Sdc-RNAi flies had significantly higher glucose levels immediately following 2 h of glucose feeding (''fed''). The difference in glucose levels between groups was still significant 2 h after the flies were again fasted (''2 h re-fast''); however, this difference disappeared after 4 h fasting (''4 h re-fast'') (Fig. 3A). Overall, these data suggested that ilp2>Sdc-RNAi flies took longer than controls to clear post-prandial glucose due to glucose intolerance.

ILP-mediated receptor activation in *Drosophila* triggers the activation of Akt through phosphorylation (Alfa and Kim, 2016), we therefore measured AKT phosphorylation levels at Ser-505 in decapitated flies at the time immediately following the ''fed'' state. We found that AKT phosphorylation was decreased by 71% in ilp2>Sdc-RNAi flies relative to control flies (Fig. 3B). This finding further corroborates that ilp2>Sdc-RNAi flies are glucose intolerant.

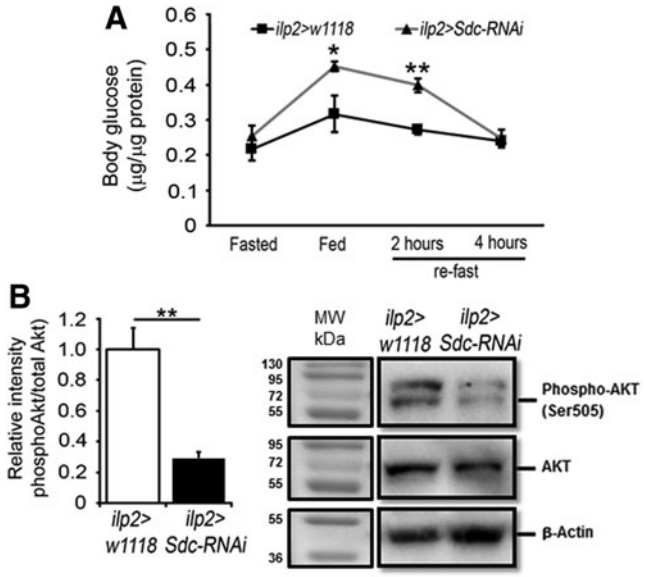

FIG. 3. IPCs-specific *Sdc* knockdown leads to glucose intolerance. **(A)** Oral glucose tolerance test data (see text for details). Values represent the means of glucose in homogenates of decapitated flies ($n = 10$). **(B)** *Left panel:* quantification of phosphorylated AKT from western blot assays following glucose feeding (''fed,'' $n = 4$). *Right panel:* representative western blots images in ilp2>w1118 and ilp2>Sdc-RNAi flies. β-actin was used as loading control. $^*p < 0.05$ and $^{**}p < 0.01$ relative to control. Error bars represent SE. MW, molecular weight.

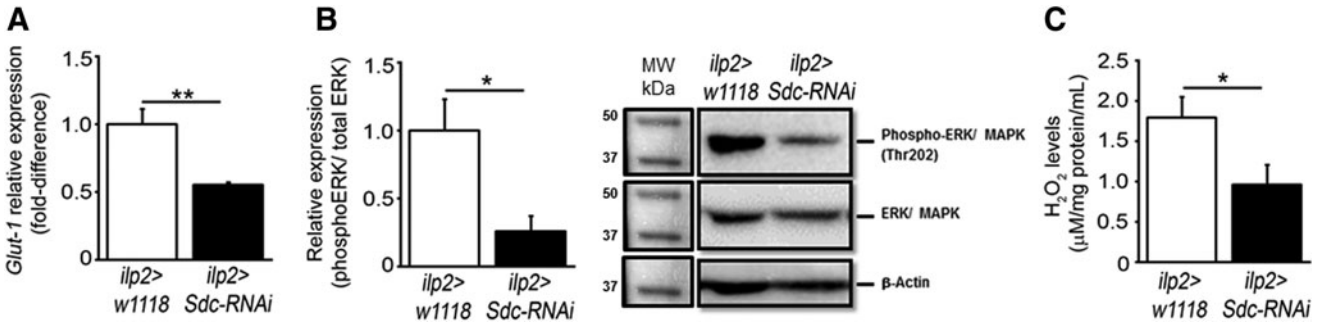

FIG. 4. IPCs-specific *Sdc* knockdown reduces levels of head *Glut1* gene expression, ERK phosphorylation, and brain reactive oxygen species. **(A)** Gene expression levels were measured by qPCR using mRNA isolated from heads of ilp2>w1118 and ilp2>Sdc-RNAi flies ($n=6$). Transcript levels of each target gene were normalized to *rp49*. **(B)** *Left panel:* quantification of phosphorylated ERK from western blot assays ($n=4$). *Right panel:* representative western blots images in ilp2>w1118 and ilp2>Sdc-RNAi flies. β-Actin was used as loading control. **(C)** Values represent the means of H_2O_2 in fly brains ($n=16$). In all panels, $*p<0.05$ and $**p<0.01$ relative to control. Error bars represent SE. ERK, extracellular signal-regulated kinase.

Knockdown of Sdc in IPCs reduces levels of head Glut1 *gene expression, ERK phosphorylation, and brain reactive oxygen species*

In mammals, the glucose-stimulated pancreatic insulin secretion (GSIS) mechanism is essential in maintaining glucose homeostasis (MacDonald *et al.*, 2005) and reduced expression of β cell *Glucose transporter* (*Glut*) genes has been associated with loss of GSIS in rodents (Unger, 1991). As mentioned in the introduction, growing evidence suggest that adult flies secrete ILPs in response to dietary glucose (Haselton and Fridell, 2010; Barry and Thummel, 2016). Additionally, it has been shown that IPCs-specific knockdown of the *Glut1* gene has no effect on *ilp2* mRNA levels, but it results in a drastic reduction in circulating ILP2 relative to controls (Park *et al.*, 2014). Based on these observations, we next compared mRNA levels of *Glut1* in the heads of ilp2>Sdc-RNAi and ilp2>w1118 flies. Notably, we found that ilp2>Sdc-RNAi had 45% less *Glut1* transcript levels compared to control animals (Fig. 4A).

Recent results of both *in vitro* and *in vivo* studies have demonstrated a role for glucose-induced ERK1/2 activation in mammalian GSIS (Longuet *et al.*, 2005; Rondas *et al.*, 2012; Bowe *et al.*, 2013; Niu *et al.*, 2016). In light of our previous work showing a link between *Drosophila* fat body Sdc and ERK phosphorylation (Eveland *et al.*, 2016), we sought to measure the phosphorylation status of ERK in the heads of ilp2>Sdc-RNAi and control flies. As predicted, we found that knockdown of *Sdc* in the IPCs led to a 75% decrease in ERK phosphorylation levels (Fig. 4B).

Reactive oxygen species (ROS), such as H_2O_2, regulate several signaling pathways, including activation of ERK in several mammalian cell types (Guyton *et al.*, 1996). Additionally, emerging data suggest that the NADPH oxidase-mediated production of H_2O_2 is involved in insulin secretion (Morgan *et al.*, 2009; Jayaram and Kowluru, 2012). Therefore, we next quantified H_2O_2 levels in the brains of ilp2>Sdc-RNAi and ilp2>w1118 flies and observed that ilp2>Sdc-RNAi had remarkably lower (46%) levels of H_2O_2 (Fig. 4C).

Discussion

Emerging data suggest that signaling by direct cell–matrix interactions plays a pivotal role in tissues involved in the control of systemic metabolism, such as adipose tissue, liver, and pancreas (Kaido *et al.*, 2006; Williams *et al.*, 2015). To this end, we previously reported that flies with a mutation in the *Sdc* gene, which encodes a transmembrane receptor that binds to matrix components (Narita *et al.*, 2004), had significantly lower *ilp2* (one of *Drosophila insulin-like peptide* genes) transcript levels in their heads than co-isogenic controls (De Luca *et al.*, 2010). Here, we show that, contrary to the results in the global *Sdc* mutant, knockdown of *Sdc* specifically in the IPCs did not have an effect on the expression of *ilp* genes. However, these flies exhibited reduced levels of circulating ILP2 protein and significant glucose intolerance compared to control flies. In line with results from previous IPC ablation studies and genetic deletion of *ilp2*, flies with reduced *Sdc* in the IPCs also had increased circulating trehalose and stored glycogen, and were more resistant to starvation conditions. Furthermore, they displayed a significant reduction in *Glut1* gene expression in their heads. This latter result is of particular interest considering that *Glut1* is a regulator of insulin secretion in *D. melanogaster* (Park *et al.*, 2014).

There are a variety of cell autonomous and nonautonomous mechanisms that have been identified to independently regulate gene expression and release of the IPCs-derived ILPs (Alfa and Kim, 2016). For instance, the expression of *ilp3* and *ilp5* is regulated by nutrient availability (Ikeya *et al.*, 2002) and reduction in *ilp3* transcript levels leads to downregulation of *ilp2* and *ilp5* expression, suggesting the presence of regulatory synergy in expression among IPCs-produced ILPs (Grönke *et al.*, 2010). Additionally, ILP6, which is synthesized in the fat body in response to nutrient availability, plays a role in regulating *ilp* expression and ILP peptide secretion in the IPCs (Bai *et al.*, 2012). The hypomorphic *Sdc* mutation significantly altered energy metabolism in flies (De Luca *et al.*, 2010). It is, therefore, plausible that the effect on *ilp2* expression previously seen in the *Sdc* mutant flies is due to an indirect cell nonautonomous signaling mechanism activated by the perturbed energy homeostasis.

Unlike previous work reporting an increase in fat storage in adult IPC knockdown flies compared to control flies (Haselton *et al.*, 2010), we did not observe any change in triacylglycerol levels between ilp2>Sdc-RNAi and ilp2>w1118 flies. This finding is not surprising. Indeed, while the effects of ILPs on *Drosophila* glucose homeostasis are well established, there are contradictory results in the literature concerning their effects

on fat storage. For example, mutations in several insulin signaling pathway members increase body fat in flies [reviewed in Ref. Teleman (2009)]. Yet, activation of insulin signaling in the fat body also leads to lipid accumulation (DiAngelo and Birnbaum, 2009). Furthermore, *in vitro* work of glucose fluxes using isotope tracers showed that insulin promotes a significant reduction in the incorporation of D-[U-^{14}C]glucose into glycogen, but it does not affect lipid synthesis in *Drosophila* (Ceddia *et al.*2003). No differences in triacylglycerol levels have been reported between flies with mutations in the single *ilp2*, *ilp3*, and *ilp5* genes and their control flies (Grönke *et al.*, 2010). Given that in this study we only measured circulating ILP2 protein, it is possible that reduction of *Sdc* transcript levels in the IPCs may alter only the release of certain ILPs.

One fascinating finding in this study is the reduced levels of H_2O_2 and ERK phosphorylation in the heads of IPCs-specific *Sdc* knockdown flies. Several studies have suggested a link between Erk1/2 activation and glucose-stimulated insulin release from mouse islets, which appears to be mediated via effects on cytoskeletal remodeling (Longuet *et al.*, 2005; Park *et al.*, 2006; Bowe *et al.*, 2013). Recently, Jayaram and Kowluru (2012) proposed a model in which glucose-induced activation of the small GTPase ADP-ribosylation factor 6 (Arf6) in β cells controls ERK1/2 activation, which in turn leads to activation of Rac1 and thereby assembly and functional activation of NADPH oxidase, an H_2O_2-generating enzyme. NADPH oxidase-generated ROS in turn facilitate cytoskeletal remodeling and insulin release (Jayaram and Kowluru, 2012). Our data indicate that this mechanism might also occur in *D. melanogaster* and that Sdc could be involved. This idea is also supported by the fact that mammalian SDC4 regulates the activity of Arf6 (Brooks *et al.*, 2012). However, one limitation of the present study is that both ERK phosphorylation and H_2O_2 levels were not assessed specifically in the IPCs. Thus, additional work is needed to exclude potential cell nonautonomous effects of IPC-specific *Sdc* knockdown on the ERK pathway and H_2O_2 production.

Conclusions

The results presented here are the first demonstration of a cell autonomous role for Sdc in insulin release from IPCs rather than in insulin production. Recent studies in mammals argue for an important role of SDC4 in pancreatic β cell function (Cheng *et al.*, 2012). Preserving the function of β cells is critical to the prevention of type 2 diabetes (Kahn *et al.*, 2014), a disease that continues to affect the population of the United States and the world at an alarming rate. Thus, further studies investigating the role of *Drosophila* Sdc in the IPCs may help elucidate the role of its mammalian counterpart in β cell function and survival.

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Disclosure Statement

No competing financial interests exist.

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Appendix 1: supplement to Materials and Methods.

1. DNA preparation.

White blood cells from blood buffy coats were used as source of DNA. 10 ml of venous blood were taken from each donor. In each blood sample to which EDTA (ethylenediaminotetraacetic acid) was added as an antioxidant was added an equal volume of TE buffer consisting of:

• NaCl 0.1 M;

• Tris HCl 0.01 M pH 7.5;

• EDTA 0.001 M.

This preliminary phase was followed by centrifugation at 4000 rpm for about 20 minutes at a constant maintained temperature of 20 ºC. Three phases have been obtained: a superior serum phase, a lower phase containing the concentrated erythrocytes and, in the interface between the two phases, the buffy coat, that is, the rich material in leukocytes which, after removing the supernatant, was withdrawn Through a Pasteur pipette. The buffy coat removed was again added TE buffer (10 ml) and subjected to further centrifugation, again at 4000 rpm for 20 minutes. At this point, the buffy coat was harvested and frozen at -20 ºC until the time the DNA was extracted.

DNA extraction by "salting out": this method is based on the physico-chemical principle, which results in a lower solubility of the macromolecules at high salt concentrations. The buffy coats were resuspended in an equal volume of erythrocyte lysis buffer to eliminate any possible erythrocytes. Such buffer is composed of:

• 155 mM ammonium chloride;

• 10 mM potassium bicarbonate;

• 0.1 mM EDTA.

The samples, kept in ice for 10 minutes, were centrifuged at 2500 rpm for 5 minutes. At the end of the centrifugation, the pellet obtained is resuspended in:

• 3 ml of buffer A (10 mM TRIS-HCl pH 8.0, 400 mM NaCl, 2 mM EDTA pH 8.0);

• 200 µl of SDS 10%;

• 500 µl of proteinase K.

The samples were left in incubation all night. The following day, 5 µl of RNAse (c = 10 mg / ml in TRIS-HCl pH 7.5) were added to each sample and left in incubation for 1 h at 37 ºC. Subsequently, 1 ml of NaCl 6 M was added to each sample, which induced the precipitation of lipids and proteins, which should already be separated by the use of SDS (sodium dodecyl

sulfate) and proteinase K. After stirring Samples for about 15 seconds, they were again centrifuged at 2500 rpm for 15 minutes.

At this point, the supernatant of each sample is transferred into sterile Falcon tubes containing an equal volume of isopronazole. A gentle agitation allowed the formation of the "flocculus" consisting of the extracted condensed DNA, which was easily extracted with the aid of a Pasteur pipette and a hook. At this point, DNA was treated with 70% ethanol and resuspended in an appropriate volume of TE buffer, consisting of:

• 0.01 M of TRIS-HCl at pH 8.0;

• 0.001 M EDTA at pH 8.0

Finally, samples were stored at 4 ° C.

To determine the concentration of extracted DNA, it was necessary to weigh the DNA present in the blood buffy coat. The DNA assay was performed with the aid of a photometer previously calibrated using a known DNA concentration solution. At this point, the previously diluted sample was subjected to absorbance (ng / µl) measurement at a wavelength of 260 nm. The absorbance value obtained indicates the sample DNA concentration.

2. Genotyping.

Real time PCR

Genotyping of the polymorphic site rs918421 (A/G) was carried out using a TaqMan Real Time PCR (SNP Genotyping kit, Applied Biosystems). The kits contain pre-designed sequence specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan MGB allele specific probes that anneal specifically to their complementary sequence between the forward and reverse primers. In both assays, the fluorescent FAM dye was used to label the more frequent allele (A), while the fluorescent VIC dye was used to label the other one (G). Sequences of primers and probes are not available. Genotyping was performed by analyzing the fluorescent pattern of each sample. PCR reactions were carried out in a total volume of 5 µl containing 20 ng of genomic DNA, 2.5 µl of TaqMan Universal Master mix (concentration of 29), 0.25 µl of Custom TaqMan SNP Genotyping Assay (concentration of 209) containing both primers and probes. The amplification protocol (60°C for 30 s and then 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min) was performed by using a step one thermal cycler (Applied Biosystems). Random regenotyping were conducted to confirm the results (Figure 1).

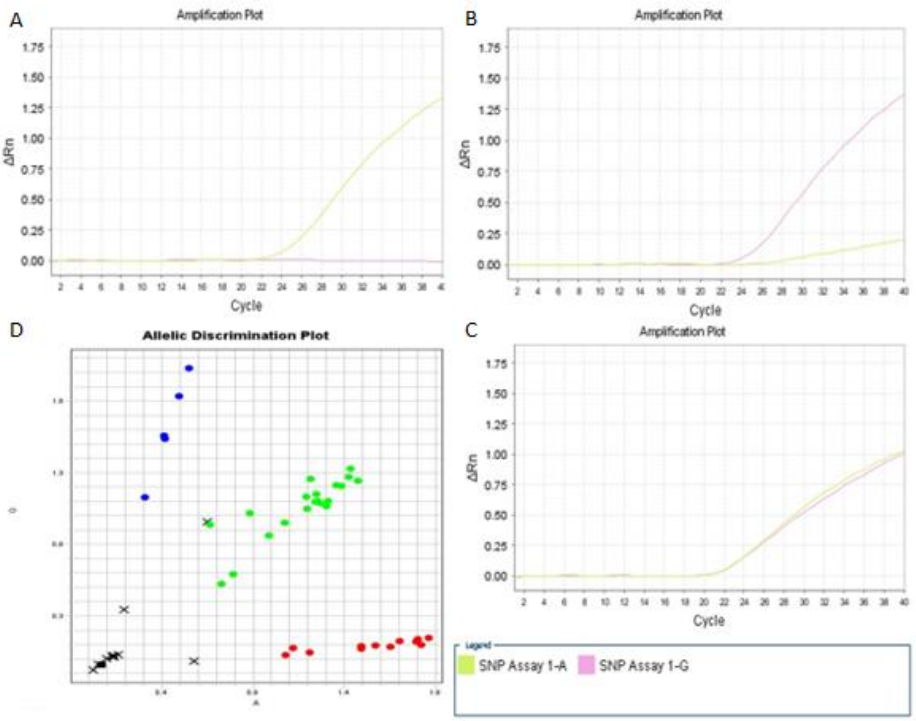

Figure 1: Genotype of SNP rs918421 in Real Time PCR. A) genotype AA only green line grows up; B) genotype GG only red line grows up; C) genotype AG both green and red lines grow up; D) genotype distribution of 48 wells Real time PCR plate.

Restriction analysis

SNPs rs14160, rs17112008, rs10056358, rs4865615 were genotyped by restriction analysis. Initially, a preliminary PCR was made (FOR and REV primers are reported in Table 1) as the amount of DNA contained in the sample is not sufficient to be analyzed; Using specific protocols, it was possible to obtain exponential amplification (up to 2^{35} times) in the region of interest.

SNP	Primer For	Primer Rev
rs14160	TCCCCTAATGGCCTCAGAGA	GCTCTCTCTCCTCACTGCTC
rs17112008	GCCTCACTTCTGCCTTGTCT	TGCCTGGTACCAAGGGAAAT
rs10056358	TCCACAGAACTCAGTGAACCA	TACTGTGCCAAAACCTGACG
rs4865615	TGCTCCAATGAGAGACACCA	TTGAGTTTATTTGGGGAACACA

Table 1: Primers sequences. The reagents were bought from SIGMA-Aldrich.

Each fragment was amplified in a final volume of 25 µl containing the reagents reported in Table 2.

Reagent	Concentration
Buffer	1X
dNTP	200 µM
Forward	0.15-0.2 µM
Reverse	0.15-0.2 µM
Taq polymerase	1 unit
DNA	0.5-0.8 µl
H2O	To bring to final volume

Table 2: The reagent used to perform the PCR belong to the REDTaq DNA polymerase kit bought from SIGMA-Aldrich and to the DreamTaq Green DNA Polymerase bought from Thermo Scientific.

The amplification protocol was performed by using the Peltier Thermal Cycler-100: pre-denaturation at 95°C for 5 min → denaturation at 95°C for 30 s → annealing at 55-60°C depending on the primers for 30 s → extension at 72°C for 30 s repeated for 35 cycles → final extension at 72°C for 10 min. The amplification of the fragments was verified through horizontal electrophoresis by loading the PCR product on 2% agarose gel and stained with ethidium bromide (reported in Figure 2).

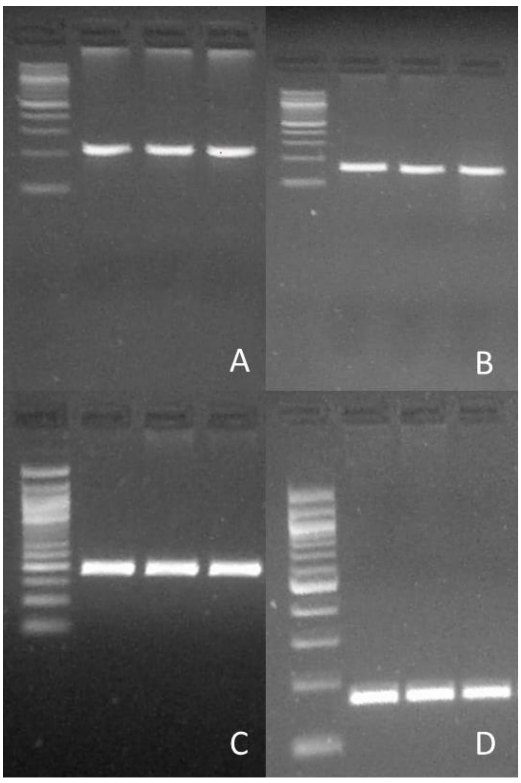

Figure 2: PCR products. A) rs14160, 227 bp; B) rs17112008, 157 bp; C) rs4865615, 193 bp; D) rs10056358, 174 bp.

An aliquot of the amplified was used to carry out an enzymatic restriction analysis. This approach has been chosen because these SNPs fall into regions and their variability creates or eliminates sites for recognition and cutting of a restriction enzyme. In general, the presence of an allele causes the enzyme identification of the sequence and it can cut the DNA in that point, while if the other allele is present, the enzyme cannot recognize it and therefore cannot cut the DNA. Specifically, the enzymes used for these assays were purchased at New England BioLabs Inc. and are reported in Table 3.

SNP	Enzyme (10,000 units/ml)	Sequence	Allele recognized
rs14160 T/C	MspA1I	CMGᵛCKG GKCᐞGMC	C
rs17112008 C/A	HpyCH4IV	Aᵛ CGT TGCᐞA	C
rs10056358 A/T	HpyCH4IV	Aᵛ CGT TGCᐞA	T
rs4865615 C/G	AluI	AGᵛCT TCᐞGA	C

Table 3: Restriction Enzymes.

To visualize the digestion result, an electrophoretic run was performed on a 2% agarose gel and stained with ethidium bromide: from the result is it possible to distinguish between the

homozygote and the heterozygote phenotypes. In Figure 3 are showed the fragments of the restriction analysis for all the SNPs.

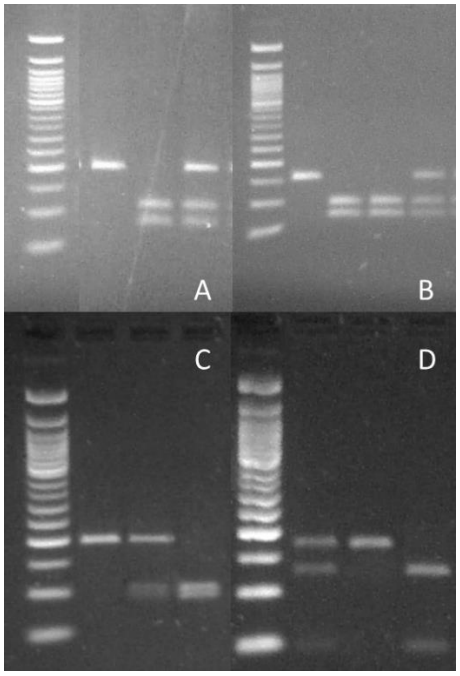

Figure 3: A) rs14160: the enzyme cuts if there is the C allele in two fragments of 97 and 130 bp. B) rs17112008: the enzyme cuts if there is the C allele in two fragments of 63 and 94 bp. C) rs4865615: the enzyme cuts if there is the C allele in two fragments of 91 and 102 bp. D) rs10056358: the enzyme cuts if there is the T allele in two fragments of 127 and 47 bp.

iPlex Gold Genotyping by Sequenom Mass Array

For all the others SNPs, the multiplex SNP genotyping was performed using polymerase chain reaction followed by primer extension and Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry using iPLEX Gold technology from Sequenom (Sequenom Inc, San Diego, CA), following the manufacturer's instructions (Figure 4).

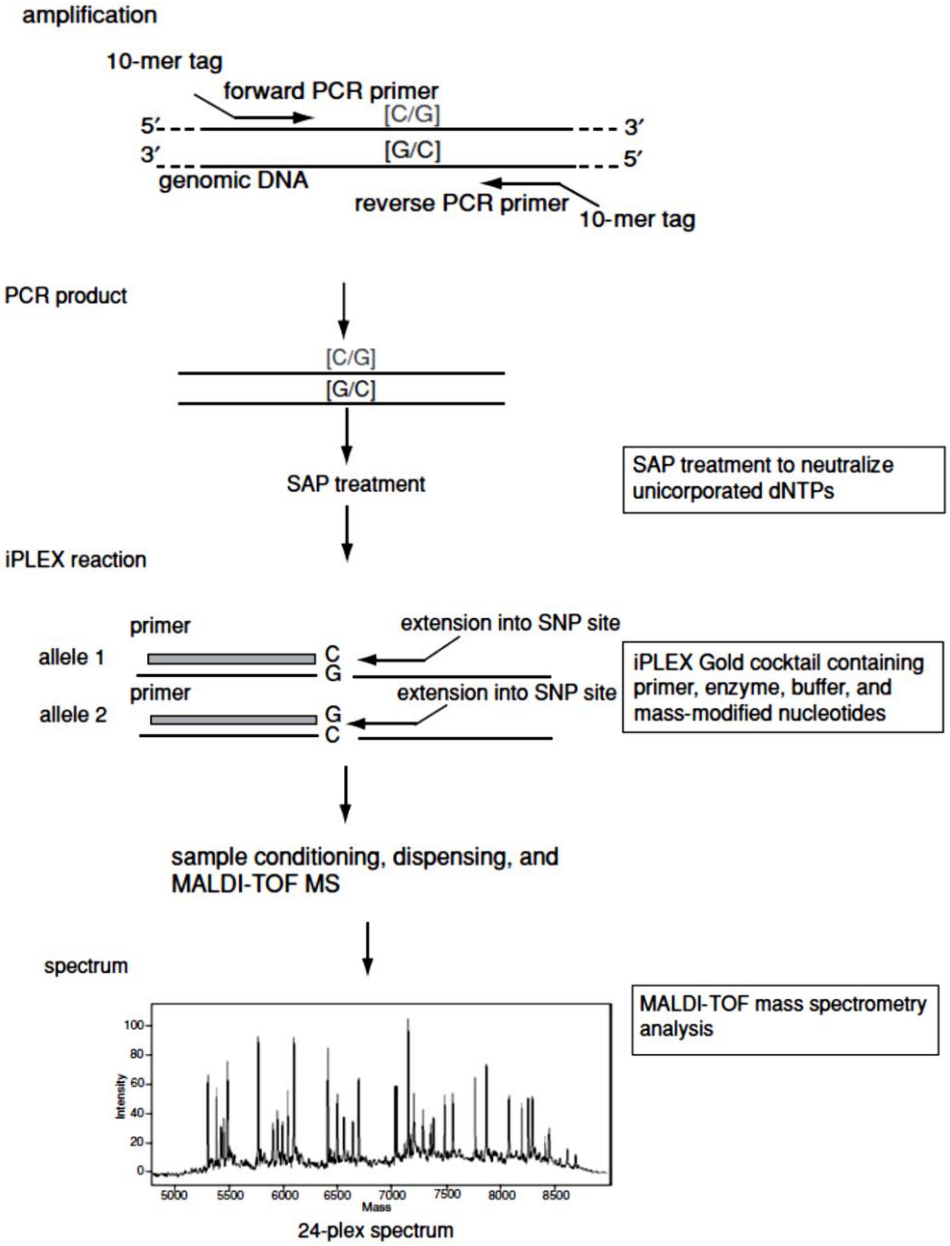

Figure 4: A schematic iPLEX reaction.

Sequenom MassARRAY Assay Designer software (version 3) was used to design primers for polymerase chain reaction and single base extension. Standard procedures were used to amplify polymerase chain reaction products: it is a multiplex PCR reaction that allow to

amplify many individual loci of DNA with minimal nonspecific by-products. The master mix and the perform thermal cycling are reported in Figure 5.

	H_2O	PCR buffer (10 ×)	$MgCl_2$	dNTP mix	Primer mix (1 µM)	HotStarTaq Plus	Total	Amount per well of 96-well plate
1 well	2.222	0.825	0.429	0.132	0.66	0.132	4.4	
1 plate	1194.55	443.52	230.63	70.96	354.82	70.96	2365.44	22 µl

1 cycle:	5 min	94°C	(initial denaturation)
45 cycles:	20 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	3 min	72°C	(final extension)
Final step:	indefinite	4°C	(hold).

Figure 5: PCR reaction.

Unincorporated nucleotides were deactivated though dephosphorylation with the shrimp alkaline phosphatase (SAP); the reaction mix and thermal cycler setup are reported in Figure 6.

	Water	10× SAP buffer	SAP (1.7 U/µl)	Total amount of mix
1 well	1.53	0.17	0.3	2
1 plate	1107.72	123.08	217.2	1448

1 cycle:	40 min	37°C
1 cycle:	10 min	85°C
Final step:	indefinite	4°C.

Figure 6: SAP reaction cleanup.

A primer extension or iPLEX reaction was subsequently implemented using the mass extension primer and the terminator. It is a universal method for detecting SNPs or small insertion/deletion polymorphisms in amplified DNA. During the reaction, the primer is extended by one mass-modified nucleotide depending on the allele and the design of the assay (Figure 7).

	H_2O	iPLEX buffer (10 ×)	iPLEX extension mix	Probe mix in 3 bins (5 μM to 15 μM)	iPLEX enzyme	Total	Amount per well of 96-well plate
1 well	0.4926	0.222	0.2	1.0444	0.041	2	
1 plate	346.79	156.29	140.8	735.26	28.86	1408	13 μl

1 cycle:		30 sec	94°C	(initial denaturation)
40 cycles:		5 sec	94°C	(denaturation)
	5 cycles:	5 sec	52°C	(annealing)
		5 sec	80°C	(extension)
1 cycle:		3 min	72°C	(final extension)
Final step:		indefinitely	4°C	(hold).

Figure 7: Primer Extend master mix with Sequenom Termination Mixes and primer extension reaction.

The clean-up step is important to optimize mass spectrometry analysis of the extended reaction product. SpectroCLEAN (Sequenom) is a cationic resin pre-treated with acid reagents. A slurry of the resin is added directly to primer extension reaction products to remove salts such as Na^+, K^+ and Mg^{2+} ions, because they can result in high background noise in the mass spectra.

The primer extension products were then spotted onto the 384-element SpectroCHIP (Sequenom) for MALDI-TOF analysis using SpectroACQUIRE v3.3.1.3 (Sequenom). Mass spectrometry helps the detection of primer extension products: the detection of an ion at the end of the tube is based on its flight time, which is proportional to the square root of its mass-to-charge ratios. Spectra were analysed using MassARRAY Typer v3.4 Software (Sequenom).

For quality control, 5% of the total number of samples was re-genotyped to assess the reliability of the genotype identification protocols. Concordance among duplicates was > 99.8% for all genotypes. For additional quality control, genotypes were excluded if Hardy-Weinberg equilibrium among controls $p < 0.05$ or call rates of < 90%.

Appendix 2: extract of the questionnaire on functional activity.

1. Activity daily living.

Activities of daily living

These questions concern what you do in your everyday life. Ignore temporary problems.

22. Feeding
    a. Do you usually feed yourself without any difficulty and completely on your own?
        ❒ Yes
        ❒ No → go to b
    b. Does someone help you to feed yourself?
        ❒ Yes → go to c
        ❒ No
    c. Since when do you have assistance to feed yourself?
        ❒ Less than a year
        ❒ Several years (up to 10)
        ❒ More than 10 years
23. Transfer
    a. Do you usually move in and out of bed without any difficulty and completely on your own?
        ❒ Yes
        ❒ No → go to b
    b. Does someone help you to move in and out of bed?
        ❒ Yes → go to c
        ❒ No
    c. Since when do you have assistance to move in and out of bed?
        ❒ Less than a year
        ❒ Several years (up to 10)
        ❒ More than 10 years

Theses questions are aimed to evaluate what the respondent actually DOES, and not what he/she is able to do.

24. Dress and undress

    a. Do you usually dress and undress yourself without any difficulty and completely on your own?

        ❒ Yes

        ❒ No → go to b

    b. Does someone help you to dress and undress yourself?

        ❒ Yes → go to c

        ❒ No

    c. Since when do you have assistance to dress and undress yourself?

        ❒ Less than a year

        ❒ Several years (up to 10)

        ❒ More than 10 years

25. Use toilets

    a. Do you usually use toilets without any difficulty and completely on your own?

        ❒ Yes

        ❒ No → go to b

    b. Does someone help you to use toilets?

        ❒ Yes → go to c

        ❒ No

    c. Since when do you have assistance to use toilets?

        ❒ Less than a year

        ❒ Several years (up to 10)

        ❒ More than 10 years

26. Bath or shower

    a. Do you usually bath or shower yourself without any difficulty and completely on your own?

        ❒ Yes

        ❒ No → go to b

    b. Does someone help you to bath or shower yourself?

❒ Yes → go to c

❒ No

c. Since when do you have assistance to bath or shower yourself?

❒ Less than a year

❒ Several years (up to 10)

❒ More than 10 years

2. Physical tests.

1. Handgrip test

The respondent is sitting; arm close to his/her body and with the elbow joint at an angle of 90° . Ask the respondent to squeeze the handle as hard as possible. Repeat the test three times with the strongest hand.

1 : …… kg

2 : …… kg

3 : …… kg

❒ The respondent is not subjected to the test, specify why: ……

2. Speed for walking 4 meters

Measure out 4 meters in a straight line by means of a string. Mark out start and stop with tape. Remove the string. Ask the respondent to stand with feet close together at the starting mark. The timing starts when you say "now". The respondent is to walk at his/her usual speed to the end mark and to stop only when he/she has passed the end marking.
The respondent can use a cane or a walker if he/she feels that it is safer.

1st walk: …….. seconds

2nd walk: ……..……seconds

❒ The respondent is not subjected to the test, specify why: ……