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THE ROLE OF GDF15 IN AGING AND AGE-RELATED DISEASES (ARDs): STUDIES ON *EX VIVO* AND *IN VITRO* MODELS

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Abstract

Aging is a complex phenomenon and represents the main risk factor for a number of diseases that because of this reason are collectively indicated as age-related diseases (ARDs). According to the Geroscience concept, aging and ARDs share the same key molecular mechanisms, suggesting that the mechanisms that drive normal aging may also lead to the onset of ARDs. In particular, it has been proposed that aging is driven by twelve biological hallmarks. Among these, two are of particular interest for our group: chronic inflammation (*inflammaging)* and mitochondrial dysfunction. These two alterations are in fact present in several ARDs. In this conceptual framework, we were interested in studying the role of Growth Differentiation Factor 15 (GDF15). This secreted protein is among the most studied molecules in the context of aging and ARDs. GDF15 is in fact one of the most upregulated circulating proteins during aging and is also strongly associated to several ARDs. GDF15 is a stress responsive protein, mainly induced by mitochondrial dysfunction, and for this reason is considered a *mitokine*. It is also induced by and associated to inflammation. The most characterized effects of GDF15 are the reduction of appetite and the regulation of food intake, exerted through the receptor GFRAL, only expressed in the central nervous system. However, GDF15 has been reported to have many different biological effects on several tissues and organs. In particular, mitochondria-protective and anti-inflammatory actions have been reported. Moreover, GDF15 also seems to be involved in cell cycle regulation and energetic metabolism regulation.

However, the precise role of GDF15 in aging and ARDs appears still unclear. The primary aim of this study was thus to analyze GDF15 expression and roles in two typical ARDs: sarcopenia and Alzheimer's disease (AD), where both mitochondrial dysfunction and inflammation are observed. To do that, we performed analyses on brain samples and cerebrospinal fluid of AD patients and controls and skeletal muscle biopsies and plasma from patients with lower limb mobility impairment (LLMI) and controls. We also used human dermal fibroblasts from subjects of different age and AD patients and two tumor cell lines (K562 and OV90), to further explore the biological effects of GDF15. In particular, we performed a gene knock-down (KD) of GDF15 in these *in vitro* models, to analyze the molecular and functional effects of GDF15 depletion in different cellular models.

As regard the study on AD, we did not find any significant difference in the CSF level of GDF15 between AD patients and non-demented controls. We found that GDF15 was expressed in different human brain areas, mainly by neurons. In frontal cortex, the ratio between the mature form of GDF15 (m-GDF15) and its precursor (pro-GDF15) was higher in AD patients and centenarians. We also observed a lower expression of OXPHOS subunits in the frontal cortex of AD patients compared to non-demented old subjects and a positive correlation between the mRNA levels of *Gdf15* and *Il6*.

Regarding the study on LLMI, we confirmed that patients with muscle atrophy had a higher plasma level of GDF15 (c-GDF15) compared to healthy subjects (HS). Surprisingly, the expression of the intramuscular precursor form of GDF15 (SM-GDF15), measured in skeletal muscle biopsies of *Vastus lateralis,* had an opposite trend, with a lower expression in LLMI compared to healthy subjects (HS). We also observed a negative correlation of c-GDF15 with IGF-1 and isometric quadriceps strength (IQS). C-GDF15 also positively correlated with IL6 in both LLMI and HS. SM-GDF15 was inversely correlated with Insulin and positively with Adiponectin. Moreover, PCA, CDA and ROC analyses showed that c-GDF15 was able to characterize LLMI patients with muscle atrophy and an inactive life-style, while SM-GDF15 discriminated HS with functional muscles and active life-style. *In vitro* data on primary dermal fibroblasts showed that the KD of GDF15 led to increased inflammation and structural and functional mitochondrial alterations. At variance, in tumor cell lines, the KD of GDF15 appears to have different effects, possibly affecting cell cycle regulation.

Overall, our data show that: i) GDF15 is expressed in human brain and in AD patients and centenarians could act as a stress responsive protein aimed at counteract mitochondrial dysfunction and inflammation associated to the disease and extreme aging; ii) GDF15 expression in skeletal muscle appears to be stimulated by myofiber contraction and is thus higher in healthy and active subjects. It could be necessary to guarantee a regular mitochondrial function in this tissue; iii) *in vitro* data on dermal fibroblasts confirmed the role of GDF15 in protecting cells from mitochondrial stress and inflammation.

Introduction

1 Aging and age-related diseases (ARDs)

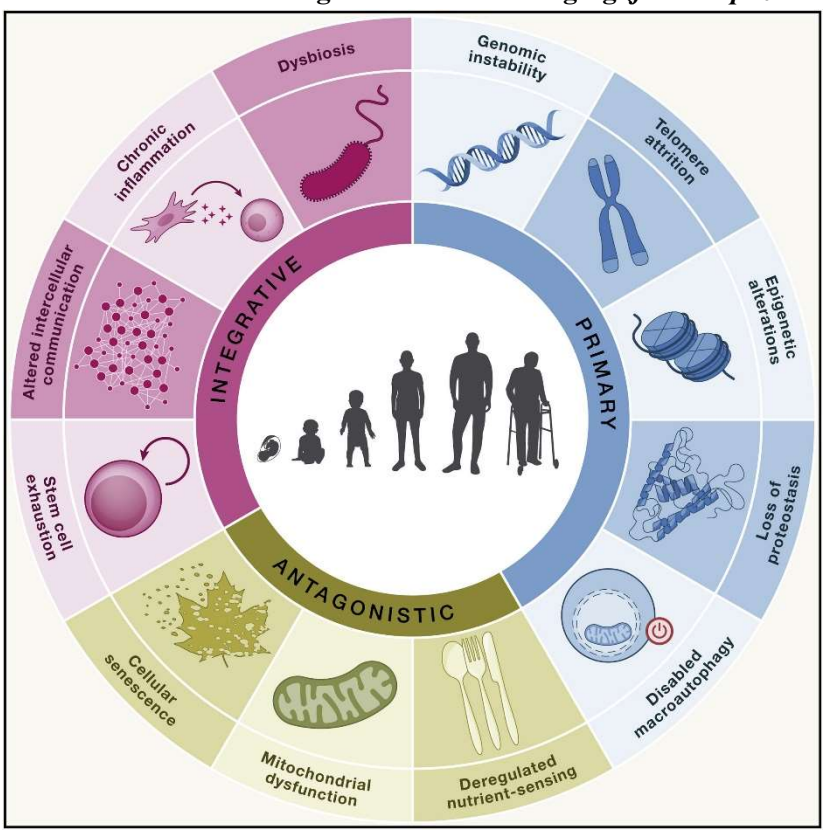
1.1 Aging and hallmarks of aging

Aging is a complex phenomenon characterized by a progressive and gradual accumulation of cell damage and loss of physiological integrity, leading to impaired function and increased vulnerability to diseases and death.

Today, aging is a major issue, considering that the world population is continuously aging. In fact, it is expected that by 2030 1 in 6 people in the world will be aged 60 years or over (about 1.4 billion people), resulting in considerable socio-economic and medical impacts (Ageing and health, World Health Organization, 2022).

From a biological point of view, aging is driven by twelve hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis (Fig. 1). All these hallmarks fulfill the following pillars: i) age-associated manifestation, ii) aging acceleration by experimentally heightening them, iii) possibility of decelerating, stopping or reversing aging through therapeutic intervention on them (López-Otín et al, 2023).

Figure 1. Schematization of the twelve biological hallmarks of aging (*from López-Otín et al, 2023*).



A detailed discussion of the twelve hallmarks of aging is beyond the scope of this thesis, but some space will be given to mitochondrial dysfunction and chronic inflammation, given the link they have with Growth Differentiation Factor 15 (GDF15), that will be discussed later (see paragraph 2).

During aging, **mitochondrial functionality** decreases due to multiple processes such as accumulation of mtDNA mutations, alterations of proteins of the respiratory chain complexes, alterations in mitochondrial dynamics and in organelles turnover. These disruptions may increase the production of reactive oxygen species (ROS) and the permeability of the mitochondrial membrane, leading to increased inflammation and triggering cell death (Amorim et al, 2022). Since many decades, such mitochondrial impairment has been proposed as a main cause of aging, according to the so-called Oxidative Stress Theory of Aging, further refined as Mitochondrial Stress Theory of Aging. According to this theory, first proposed by Denham Harman in 1950s, aging is the result of the accumulation of oxidative damage, closely linked to ROS production. In 1989, Linnane and coworkers refined the theory postulating that, more in general, mitochondrial dysfunction and the accumulation of mtDNA mutations are the main contributors to the aging process. (Liu et al, 2014; Linnane et al, 1989). Interventions on mitochondrial function have shown to delay aging, however this theory has been put under scrutiny, since ROS production is needed for cell communication, and its abolishing does not promote lifespan extension (Doonan et al, 2008), and, contrary to expectations of this theory, an induction of a mild mitochondrial stress is able to improve lifespan in model organisms (López-Otín et al, 2023; Ristow and Zarse, 2010; Rose et al., 2017). This phenomenon is known as “mitohormesis” and relies on the production of mitochondrial metabolites, ROS, peptides and **mitokines** (*i.e.* cytokines produced in response to mitochondrial stress) that are able to induce a stress-compensatory response with beneficial effects on the whole organism (Durieux et al, 2011; Bárcena et al, 2018).

A state of chronic and low-grade **inflammation** is closely associated to aging and is known as inflammaging (Franceschi et al, 2000). In fact, the level of typical inflammatory mediators, such as Interleukin 6 (IL6), Tumor necrosis factor α (TNFα) and C-reactive protein (CRP), increases during aging (Morrisette-Thomas, 2014; Wyczalkowska-Tomasik et al, 2016). In addition, a shift in T cells population is observed, with a hyperfunction of pro-inflammatory Th1 and Th17 cells, accompanied by defective immunosurveillance, lost of self-tolerance and reduced repair of biological barriers (Carrasco et al, 2022). The chronic presence of inflammation during aging becomes detrimental, contributing to age-related cellular and molecular damages. The main factors that promote inflammaging include the increased production/decreased clearance of cellular debris and misfolded/misplaced proteins, accumulation of senescent cells and the release of damage associated

molecular patterns (DAMPs) from dysfunctional mitochondria, closely linking mitochondrial dysfunction and inflammation (Franceschi et al, 2000, 2018; Picca et al, 2017; Conte et al, 2020).
Notably, inflammaging has also a counterpart known as 'anti-inflammaging', whose mechanisms and mediators are still under investigation, although some of them have possibly been identified and include the mitokines [GDF15, Fibroblast Growth Factor 21 (FGF21), Humanin] (Conte et al, 2019, 2020).
Not only mitochondrial dysfunction and inflammation are interrelated, but so are all the twelve hallmarks of aging. There is a complex interconnection between the hallmarks of aging, starting from the primary (which reflect damages affecting the genome, telomeres, epigenome, proteome, and organelles) (Fig. 1) that determine the pace of aging (López-Otín et al, 2023).

1.2 Biomarkers of aging

Aging is a heterogeneous process and chronological age does not always represent accurately the intra-individual variability. For this reason, the scientific community is constantly searching for biological markers of aging, which can better represent and quantify aging progression and processes, by assessing the biological age. Biological age is defined as an individual's age defined by the level of age-dependent biological changes, such as molecular and cellular damage accumulation. In practical use, this is often summarized as a number (in units of time) matching the chronological age where the average person in a reference population shares the individual's level of age-dependent biological changes (Moqri et al, 2023). Biological age is an estimate resulting by a single marker or by the combination of different markers, which correlates with chronological age and is also capable to distinguish individuals that are "younger" or "older" compared to their chronological age (Franceschi et al, 2018). Ideally, a biomarker of aging should be a measurable feature, able to predict future health and survival better then chronological age. Moreover, it should be reproducible and the measurement should cause minimal or no- trauma for the patients. Biomarkers of aging can be based on routine laboratory measurements, phenotypic data or molecular data derived from high throughput analyses ("omics analyses") (Hartmann et al, 2021).
Considering that aging is a multi-factorial and complex process, to date, no single biomarker or small set of markers is considered sufficient to provide a valid measure of biological aging. Therefore, it remains a main goal for scientists to provide a more accurate measure of biological age or predictors of the onset of age-related diseases (ARDs) (Wagner et al, 2016).
Different biomarkers of aging have been proposed, for example: DNA methylation markers, anthropometric markers, blood-based and molecular markers (Franceschi et al, 2018; Wagner et al, 2016; Salvioli et al, 2023). A large number of epigenetic clocks based on **DNA methylation** have

been proposed starting from 2005, when Fraga and coworkers (2005) showed that in human DNA, methylation strongly changes during aging. Subsequently, DNA methylation has been proved to correlate with chronological age and different clinical conditions (Franceschi et al, 2018; Bacalini et al, 2015), and now various clocks based on the methylation of different CpG sites in the genome are widely used to determine biological age.

As regards **anthropometric markers**, many of them are widely used to assess the progression of biological aging. Even if these markers are not as accurate as the epigenetic and molecular ones, they are easy, rapid and cheap to measure in large cohorts of subjects (Wagner et al, 2016). In particular, walking speed, handgrip strength and chair stand are frequently used for monitoring aging progression. A weaker handgrip strength, slower walking speed and less repetition in chair stand test are all associated with greater mortality rates (Cooper et al, 2010; Wagner et al, 2016). In addition, considering that aging is also characterized by increased fat mass and reduced lean mass, also parameters that measure body composition are useful to assess the pace of aging. For example, a higher BMI is associated to higher mortality and is a risk factor for cognitive decline (de Hollander et al, 2012; Whitlock et al, 2009).

Finally, different blood-based and **molecular biomarkers** are used to estimate biological age. The majority of these markers is related to cardiovascular diseases, inflammation, glucose metabolism and nutritional status. Different meta-analyses showed that the lipid profile is a strong predictor of mortality and morbidity (Briel et al, 2009; Sarwar et al, 2007). A large number of inflammaging peptides are used as biomarkers of aging, such as IL6, IL1β, TNFα, CRP. For example, CRP has been found to be a strong predictor of all-cause and specific mortality (Franceschi et al, 2000; Kaptoge et al, 2010; Salvioli et al, 2023). Interestingly, centenarians, that shows extreme longevity and delayed onset of age-related diseases, presents high levels of pro-inflammatory cytokines such as IL6, but are also characterized by high levels of anti-inflammatory cytokines like IL10 and transforming growth factor beta (TGF-β). These anti-inflammatory molecules likely counteract the effect of the pro-inflammatory ones (Salvioli et al, 2009).

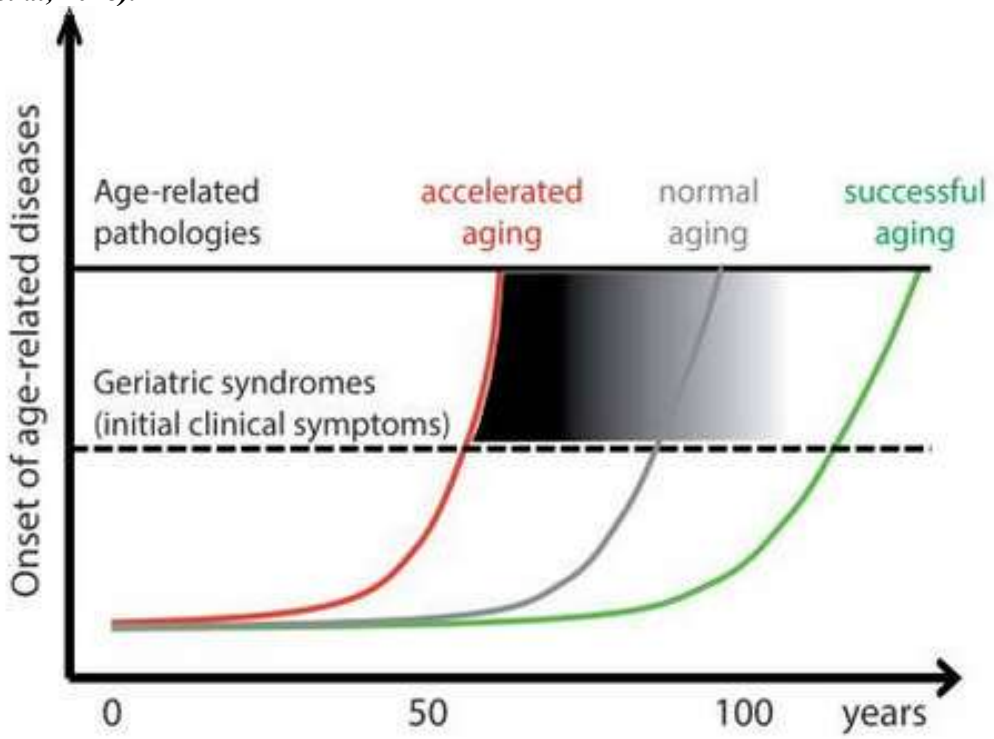
Recently, advances in proteomic analysis techniques have made it possible to identify other circulating biomarkers of aging. A recent study, showed that it is possible to build a "proteomic clock" to measure biological age by measuring the levels of 15 circulating proteins, highlighting their potential as biomarkers of aging processes (Coenen et al, 2023). One of these proteins is **GDF15**, which several studies have shown to be one of the most upregulated protein during aging. GDF15 is also strongly associated with overall mortality and many age-related clinical conditions (Tanaka et al, 2018; Conte et al, 2019; Liu et al, 2021; Guo et al, 2024).

Furthermore, Wyss-Coray's group recently showed that by measuring organ-specific circulating proteins, it is possible to measure organ-specific aging differences and that diseases that affects specific organs are related to faster aging of those organs (Oh et al, 2023).

1.3 The *continuum* of aging and ARDs

Aging is the main risk factor for the most common diseases of developed countries: cardiovascular diseases, cancer and neurodegeneration. These are also the prevalent ARDs. (Niccoli and Partridge, 2012). Many studies on model organisms have shown that intervention that prevent or delay aging are also able to prevent or delay the onset of ARDs, consolidating the idea put forward by the newborn discipline Geroscience that aging and ARDs largely share the same molecular mechanisms and thus must not be considered as two separate processes, but rather a *continuum* without clear boundaries (Franceschi et al, 2018). According to the *continuum* hypothesis, in individuals in whom ARDs occur early, an accelerated aging process takes place. On the contrary, in individuals in whom these diseases appear very late or not at all, such as centenarians, it appears that the pace of aging is much slower, and thus they represent a model of healthy aging (Collino et al, 2013; Franceschi et al, 2018). These different aging trajectories depend on the complex interaction between genetics, epigenetics, environment and life-style on which the rate of adaptation and response to lifelong stressors depends (Franceschi et al, 2018; Santoro et al, 2020) (Fig. 2).

Figure 2. Different genetic and epigenetic predispositions and different life-styles determine different aging trajectories, with early or delayed onset of ARDs, according to the *continuum* hypothesis (*from Franceschi et al, 2018).*



The *continuum* hypothesis of aging is fundamentally based on the fact that aging and ARDs share the same basic molecular and cellular mechanisms, that are also considered the "seven pillars" of aging: adaptation to stress, loss of proteostasis, stem cell exhaustion, metabolism derangement, macromolecular damage, epigenetic modifications and inflammation (Kennedy et al, 2014).

Two important examples of widespread ARDs are Alzheimer's disease and sarcopenia. A brief description of these two ARDs will follow.

Alzheimer's disease (AD) is the most common neurodegenerative disorder. It is characterized by loss of neurons and synaptic connections. Some of the main features of the physiopathology of AD are the deposition of amyloid-beta (Aβ) plaques, and neurofibrillary tangles of phosphorylated Tau protein (Pradeepkiran and Reddy, 2020; Wang et al., 2020). The first brain areas affected by the disease are the entorhinal cortex and the hippocampus, then, the alterations spread to the parietal, temporal, and frontal lobes (Braak et al., 2006). The molecular mechanisms that underly and cause the onset of AD are not yet completely clarified, but some of them are thought to be mitochondrial dysfunction, oxidative stress and neuroinflammation (Pradeepkiran and Reddy, 2020; Wang et al., 2020; Chiariello et al, 2023).

Sarcopenia is a musculoskeletal disease, defined by the progressive loss of muscle mass and strength, in particular in older subjects (Cruz-Jentoft et al, 2019). The causes of sarcopenia are not yet completely understood but it is thought to be a multifactorial disease, in which hormone changes, neurological decline, increased inflammation, decline in physical activity and poor nutrition all together contribute to its onset (Walston, 2012). Sarcopenia is becoming highly prevalent in the elderly population and it has a remarkable impact on the on the quality of life, since it is associated with increase of falls, poor physical function and difficulties in activities of daily living. In addition, it is considered both a precursor and a physical manifestation of frailty and is often difficult to diagnose (Wilson et al, 2017; Veronese et al, 2019).

2 Growth Differentiation Factor 15 (GDF15)

GDF15 is a distant member of the TGF-β superfamily. It was originally discovered almost 30 years ago independently by three research groups and first described as macrophage inhibitory cytokine-1 (MIC-1), Placental Transforming Growth Factor Beta (PTGFB) and Placental bone morphogenetic protein (PLAB) (Bootcov et al, 1997; Hromas et al, 1997; Lawton et al, 1997).

GDF15 is a stress-response protein, mainly induced in response to **mitochondrial** stress, but also to **inflammation** and as a part of the mitochondrial unfolded protein response (UPRmt). Its production has positive effects on health and lifespan of model organisms and, as briefly mentioned before, for this reason GDF15 perfectly suits the definition of mitokine as provided by Dillin and colleagues in

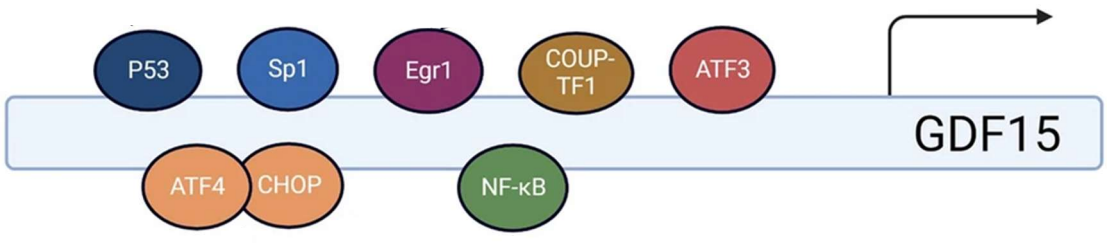
2011 (see paragraph 1.1) (Durieux et al, 2011). In apparent contrast to this role, the expression of GDF15 is really low in young and healthy subjects and dramatically increases with age and chronic or acute states of illness. In particular, the levels of GDF15 are elevated in different ARDs, such as cardiovascular diseases, type 2 diabetes and many types of cancer (Conte et al, 2022). Moreover, it is considered a marker of mitochondrial diseases (Yatsuga et al, 2015, Montero et al, 2016).

GDF15 is expressed at low levels in several tissues and at higher levels in placenta, prostate and urinary tissues and is also found in the bloodstream. Even if its level is strongly associated with aging and it has been proposed as a biomarker of aging, the exact pathophysiological roles of GDF15 in the aging process are not completely understood. In particular, it is not yet clear whether increased levels of this mitokine in old age have detrimental or beneficial/protective effects on the organisms (Conte et al, 2022).

2.1 Transcriptional regulation of GDF15

GDF15 transcriptional regulation is very complex. In fact, its promoter contains different cis- and trans- acting elements that allow a fine regulation of the gene expression, in response to a wide range of cellular stresses (Conte et al, 2022; Wan and Fu, 2024). Several transcription factors (TFs) act by binding these elements. The main TFs that regulate GDF15 gene expression are: p53, ATF3, ATF4, Chop, XBP1, NF-κB, Sp1 and Sp3, EGR1 (Conte et al, 2022) (Fig. 3). While the majority of these TFs are thought to regulate GDF15 expression in response to specific types of stress or pathological conditions, Sp1 and Sp3 appear to be important in regulating the basal transcription of GDF15 (Baek et al, 2001).

Figure 3. GDF15 promoter contains different cis- and trans- acting elements that allow a fine regulation through the action of several transcription factors (*adapted from Wan and Fu, 2024).*



A more detailed description of the TFs of GDF15 more associated with aging will follow.

p53

GDF15 promoter presents two different binding sites for p53, known as p53-type response elements, (RE1 and RE2) (Osada et al, 2007). Different studies showed that p53 is responsible of the up-

regulation of GDF15 observed in various types of cancer, where it may act by reducing cell proliferation, invasion and tumor progression, thus suggesting that a part of p53 tumor suppressing actions may be exerted through GDF15 (Tsui et al, 2015; Kang et al, 2013; Conte et al, 2022).

A specific interaction between p53 and GDF15 during aging has not been explored, however, p53 is responsive to oxidative stress, which in turn can elicit a mitochondrial dysfunction, both reported to increase with age. It is therefore possible that an increased activation of p53 in response to those stresses might also impinge upon GDF15 transcriptional activation (Conte et al, 2022). Another possibility is an inflammation-driven activation; it is in fact reported that C-Reactive Protein can induce GDF15 via p53 binding to RE1 and RE2 (Kim et al, 2018).

ATF3, ATF4, Chop, XBP1

GDF15 gene expression is also regulated by the integrated stress response (ISR) pathway. The ISR is a complex signaling pathway that aims to restore the homeostasis of the cells in response to different stresses, such as oxidative stress, hypoxia and nutrient deprivation. These stresses generally cause an accumulation of misfolded proteins in the endoplasmic reticulum (ER), that is then able to trigger the ISR (Costa-Mattioli and Walter, 2020). In particular, ER stress leads to the phosphorylation of the eukaryotic translation initiation factor 2 (eIF2) which in turn can activate the translation of the mRNA of ATF4. ATF4, together with ATF3 and C/EBP homologous protein (Chop), and functional spliced x-box binding protein 1 (XBP1) are considered the key TFs of the ISR and are also involved in the UPRmt. In addition, these proteins are directly responsible of the upregulation of GDF15 gene expression (Day et al, 2019; Lee et al, 2010; Patel et al, 2019; Conte et al, 2022).

The precise connection between these TFs and GDF15 during aging is still unclear, however, several findings showed that they could play a key role in the aging process. For example, it has been shown that the overexpression of XBP1 in *C. elegans* expands lifespan (Martínez et al, 2017) while its conditional knock-out (KO) in mice accelerates age-related neurodegeneration (McLaughlin et al, 2018).

For ATF3, ATF4 and Chop contrasting data emerge from literature. It has been reported that elevated levels of these three proteins were present in tissues of slow-aging mouse model compared to wild type (Li and Miller, 2014). On the other hand, other studies indicated that the levels of ATF3, ATF4 and Chop were elevated in cellular and animal models of aging (Ghosh et al, 2015; Liu et al, 2020).

NF-κB

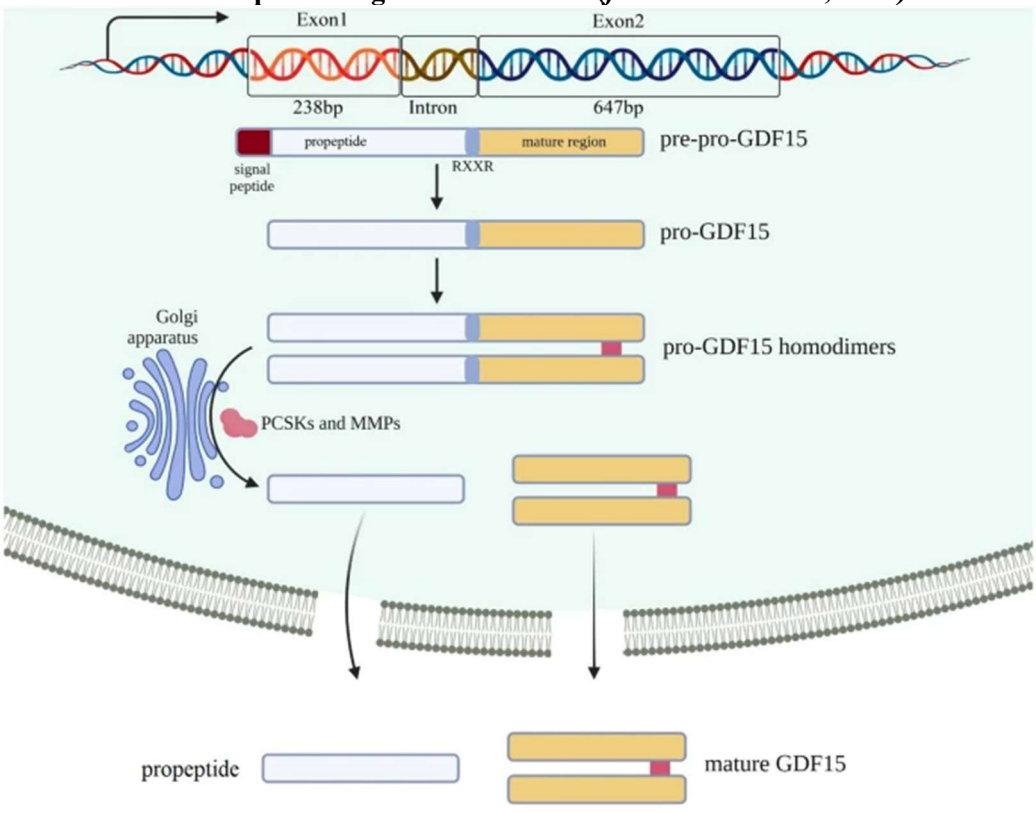
GDF15 gene expression is also regulated by NF-κB. This TF is well known as a regulator of different pro-inflammatory genes, being involved in inflammation, cell proliferation and immune response. It has been shown that a potential NF-κB-GDF15 axis could be involved in cell escape from

macrophage activity in the first stages of tumorigenesis (Ratnam et al, 2017). Considering that NF-κB is a key TF, that may act as a driver of aging, it could be involved in the upregulation of GDF15 observed in aging (García-García et al, 2021). Moreover, this TF is associated to several ARDs by the activation of the expression of senescence-associated secretory phenotype (SASP) genes. GDF15 has been recently included in the list of SASP (Di Micco et al, 2021), and is therefore possible that it is regulated by NF-κB, together with other SASP (Conte et al, 2022).

2.2 GDF15 post-translational processing and secretion

In addition to the transcriptional regulation, GDF15 is also subject to a complex regulation at protein level. Human GDF15 is a highly conserved gene (2.7 kb), composed of two exons and one intron and located on chromosome 19p13.1-13.2, that encodes for a 308 amino acid protein. Like many other proteins, GDF15 is first synthesized as a biologically inactive precursor (pre-pro-GDF15), containing a N-terminal signal peptide, that is important for its intracellular trafficking and secretion. After removal of the signal peptide, the remaining peptide (pro-GDF15) dimerizes to form the pro-GDF15 homo-dimer. This dimer is then cleaved at a conserved furine-like cleavage site (RXXR), forming the C-terminal mature GDF15 dimer (m-GDF15), bound by a single disulfide bond. This homodimer is the form of GDF15 that is most secreted and is found in the bloodstream (Conte et al, 2022; Li et al, 2018) (Fig. 4).

Figure 4. Overview of GDF15 processing and maturation (*from Wan and Fu, 2024)*



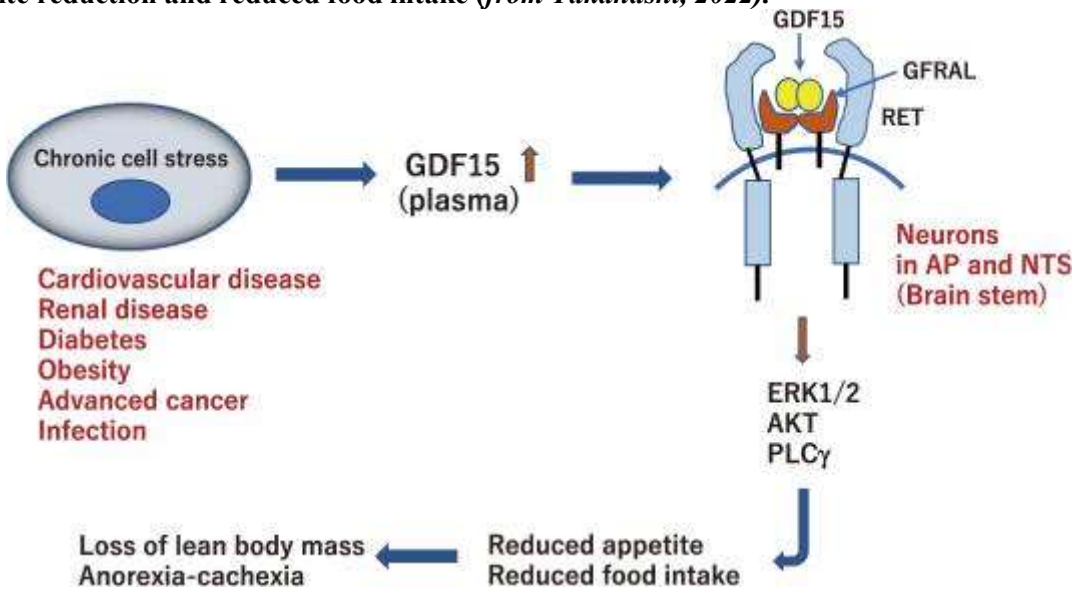
Interestingly, it has been found that pro-GDF15 is also secreted. In fact, a study found that different tumor lines secrete pro-GDF15, which in turn is able to bind the extracellular matrix (ECM). This ECM-bound pro-GDF15 constitutes a storage of GDF15, making the propeptide a regulator of the balance between ECM stores and circulating serum levels of GDF15. Furthermore, the ECM association of pro-GDF15 is thought to play a central role in modulating the local bioavailability of GDF15 in particular conditions, such as specific tumor microenvironments (Bauskin et al, 2005).
The main proteases known to cleave pro-GDF15 into the mature form belong to the family of the proprotein convertase subtilisin/kexin (PCSK) serine protease, and are PCSK3, PCSK5 and PCSK6. These three proteases act specifically at the RXXR cleavage site (Li et al, 2018). The role of these proteins on GDF15 maturation has been proven experimentally; in fact, the inhibition of the PCSKs led to a decrease of the extracellular levels of the mature form of GDF15 (Li et al, 2018). These enzymes are widely expressed in different tissues and literature data proposed a role in several ARDs (Choi and Korstanje, 2013; Chen et al, 2019; Conte et al, 2022).
In addition to PCSKs enzymes, during the early stages of development pro-GDF15 is also processed by matrix metalloprotease 26 (MMP26) (Wang et al, 2021).
Recently, it has been observed that the maturation of GDF15 is likely to happen in the Golgi apparatus, probably through the action of the β-arrestin1 (ARRB1), that binds the pro-GDF15 and facilitates its transport to the Golgi apparatus to promote the subsequent GDF15 cleavage and maturation through PCSK enzymes (Zhang et al, 2020; Conte et al, 2022) (Fig. 4).
Interestingly, it has been observed that pro-GDF15 may contain a non-canonical nuclear localization signal domain and could thus be imported in the nucleus, where it may act to regulate the expression of genes related to the Smad pathway. However, it should be noted that, although this hypothesis is particularly intriguing, to date only one scientific article supports it (Min et al, 2016).

2.3 Receptor(s), signaling and biological effects of GDF15

The exact mechanisms of action of GDF15 are still uncertain. First of all, the receptor (or receptors), by which GDF15 exerts its actions are not yet fully defined. To date, the only known GDF15 specific receptor is the GDNF α-like receptor (**GFRAL**). This receptor has been identified in 2017 simultaneously by four research groups. To work, it requires the action of the co-receptor REarranged during Transfection (RET). While RET is widely diffused across different tissues, GFRAL is believed to be only expressed in two small hindbrain centers involved in appetite control: the nucleus of the solitary tract (NTS) and the area postrema (AP) (Emmerson et al, 2017; Hsu et al, 2017; Mullican et al, 2017; Yang et al, 2017). The interaction of GDF15 with GFRAL leads to the auto-phosphorylation

of the intracellular domain of RET, then it activates different signaling pathways, in particular ERK1/2, Akt, FOS, and PLC-γ (Takahashi, 2022) (Fig. 5).

Figure 5. GDF15 acts by binding the receptor GFRAL in the NTS and AP and recruiting the co-receptor RET. This interaction activates different signaling pathways, such as ERK1/2, Akt and PLC-γ, leading to appetite reduction and reduced food intake (*from Takahashi, 2022).*



GDF15-GFRAL axis is mainly involved in energy metabolism and body weight regulation and these are the best-defined biological effects of GDF15. These activities are exerted through a non-homeostatic pathway, not associated to hormone regulation but rather regulated by stress-related responses that are able to induce GDF15 expression (Hsu et al, 2017). It has been observed that high levels of GDF15 led to loss of body weight in different human pathological conditions, via the activation of GFRAL-positive neurons. Moreover, GDF15 is one of the main drivers of cancer cachexia (Hsu et al, 2017; Conte et al, 2022). Accordingly, studies in model animals found that GFRAL KO mice were resistant to the anorexigenic action of GDF15, both under stress conditions and after chemotherapy, thus confirming the role of the GDF15-GFRAL axis in body weight regulation. In agreement, monoclonal antibodies that antagonize GDF15 are able to inhibit tumor-induced cachexia. Another study found that GDF15, in association with leptin, induced in mice a strong neuronal activation in the AP, leading to body weight and adiposity reduction (Hsu et al, 2017; Suriben et al, 2020; Conte et al, 2022; Breit et al, 2023). Interestingly, recent studies showed that GDF15 level was increased after acute starvation and that GDF15 was important for maintaining energy expenditure during calorie restriction (Chrysafi et al, 2024; Wang et al, 2023). The anorexigenic action of GDF15 is likely to be driven by the activation of the neurons of the parabrachial nuclei, known to regulate food intake (Emmerson et al, 2017). Moreover, a recent study showed that fetal-derived GDF15 is the main responsible of nausea and vomit during pregnancy, and

in particular of its most severe form hyperemesis gravidarum, confirming the link between GDF15, food intake regulation and food aversion (Fejzo et al, 2024).

Other potential central actions of GDF15 have been reported. In particular, it seems that GDF15 may increase lipolysis in adipose tissue via the sympathetic nervous system, in a brain-somatic tissue circuit. In addition, GDF15 probably reduces gastric motility by acting on the vagus nerve or the dorsal motor nuclei (Breit et al, 2020).

Several peripheric actions of GDF15, not dependent on the activation of neurons in the AP and NTS, have also been reported. Different studies proposed **anti-inflammatory** effects of GDF15. It has been shown that GDF15 reduces the expression of pro-inflammatory cytokines and prevents the activation of T cells in the liver of mice with fibrosis. Moreover, GDF15 KO induces the overexpression of TNF-α by T cells and the activation of these cells, thus worsening liver fibrosis and injury (Chung et al, 2017). Abulizi and colleagues (2017) found that GDF15 KO in mice caused increased inflammation in response to treatment with lipopolysaccharide (LPS) while transgenic mice overexpressing GDF15 were protected from LPS-induced organ injury (Abulizi et al, 2017). Another important work reported that GDF15 was overexpressed in patients with sepsis compared to healthy subjects. Moreover, they found that GDF15 activity was fundamental in mice for surviving both bacterial and viral infections, suggesting a key role as a central mediator of tissue tolerance to inflammation (Luan et al, 2019).

It should be noted that, in contrast to what has just been illustrated, many studies proposed a pro-inflammatory effect of GDF15. For example, a study showed an involvement of GDF15 in the development of atherosclerosis, by regulating apoptotic cell death and IL6-dependent inflammatory response (Bonaterra et al, 2012). As mentioned before, other studies identified GDF15 as a component of the SASP and a marker of cellular **senescence** (Di Micco et al, 2021). These contrasting findings confirm the complexity around GDF15 biology.

As already mentioned, GDF15 is considered a marker of **mitochondrial dysfunction** and has also been proposed as marker of mitochondrial diseases, as its expression strongly increases in these pathologies (Montero et al, 2016). A study that evaluated different putative biomarkers of mitochondrial diseases, including FGF21, lactate, pyruvate and creatine kinase, found that circulating GDF15 was the most sensitive and specific marker, being useful not only for the diagnosis of mitochondrial diseases but also for the evaluation of disease's severity (Yatsuga et al, 2015). A work of Chung et al (2017) found that, in mice, a muscle-specific deletion of *Crif1*, that causes an OXPHOS dysfunction, led to an overexpression of GDF15 mediated by Chop. GDF15 high levels determined in turn an improvement of glucose utilization and adiposity (Chung et al, 2017). Accordingly, a recent study showed that GDF15 knock-down (KD) in human dermal fibroblasts caused reduced basal and

ATP-coupled respiration, an increased number of elongated and damaged mitochondria and the overexpression of different SASP, such as MMP3 and MMP10, suggesting a role for GDF15 in the induction of cell senescence and in maintaining functional and intact mitochondria (Wedel et al, 2023).

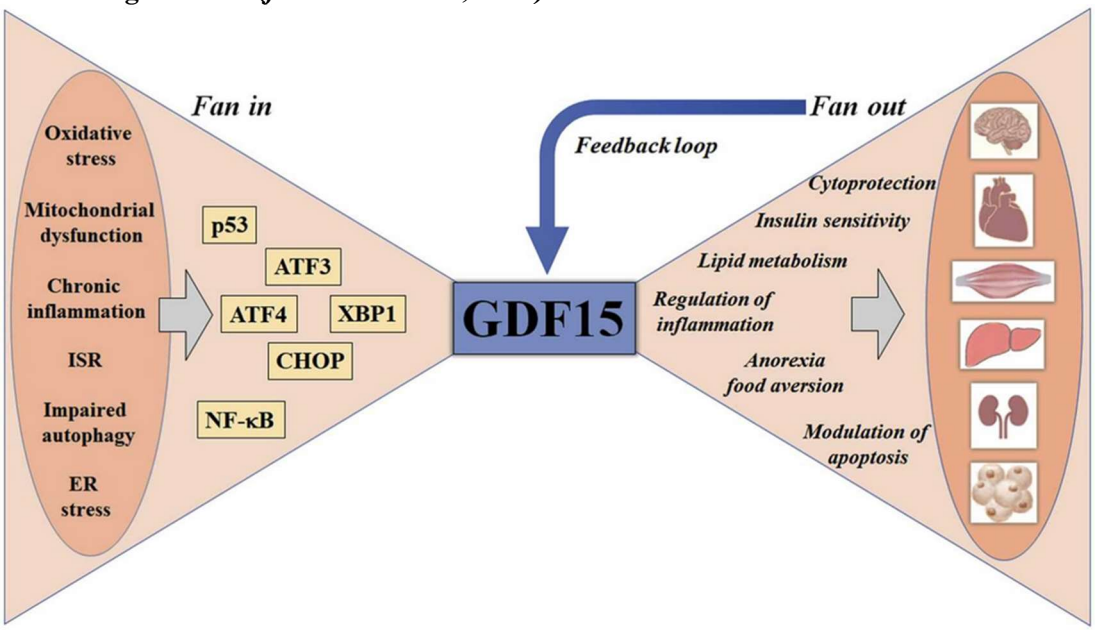
Several studies showed that GDF15 also acts on peripheral tissues by increasing lipolysis, oxidative **metabolism** and thermogenesis, reducing insulin sensitivity. In particular, mice overexpressing GDF15 showed reduced adiposity, increased resistance to obesity, lower serum level of insulin, IGF1 and leptin, compared to WT mice. These effects seem to be due to an increased expression of genes involved in oxidative metabolism, lipolysis and thermogenesis, such as UCP1, PGC1α, ATGL and HSL (Chrysovergis et al, 2014; Conte et al, 2022). In agreement, the genetic ablation of GDF15 in UCP1 transgenic mice induced a progressive increase in body fat mass and elevated levels of plasma leptin (Ost et al, 2020).

Finally, a role of GDF15 in the regulation of **cell cycle** and cell proliferation has been proposed. In particular, GDF15 treatment increased the cell proliferation rate of human umbilical vein endothelial cells (HUVECs) by enhancing the expression of G1 cyclins, cyclins D1 and E, through the PI3K/Akt, ERK, and JNK pathways (Jin et al, 2012). Other studies confirmed a GDF15 effect in promoting cell proliferation and survival, acting as a mitogenic and anti-apoptotic factor (Conte et al, 2022). For instance, a study showed that GDF15 could protect cardiomyocytes against different pro-apoptotic stimuli via the kinases PI3K and ERK and the action of the transcription factor R-SMAD1 (Heger et al, 2010).

All the actions and effects of GDF15 on peripheral tissues seem to be in contrast to the reported highly specialized localization of GFRAL in AP and NTS. In this regard, two hypotheses have been proposed: i) a splicing variant of GFRAL that lacks the transmembrane domain, but contains the GDF15 binding one, has been observed. It is possible that this truncated variant of GFRAL acts as a soluble form of the receptor, able to bind circulating GDF15 and trigger the signaling cascade in several peripheral tissues together with the action of the co-receptor RET, that is ubiquitously expressed (Li et al, 2005); ii) GDF15 may act through TGF-β receptors, in particular ALK5 and TGF-β type II receptors (Artz et al, 2016). Furthermore, a very recent immunohistochemical study performed in mice provides evidence for a more widespread expression of GFRAL. In particular, they found that, at least in mice, GFRAL was expressed also in prefrontal cortex, hippocampus, liver, small intestine, adipose tissue, kidney and muscle (Fichtner et al, 2024). More evidence and data, especially in human tissues, are required to confirm this hypothesis, but a wider expression of GFRAL could explain, at least in part, the pleiotropic biological effects of GDF15.

Considering the elevated complexity in the biology of GDF15, the involvement of several TFs in its regulation and the different effects that it has, GDF15 could be seen as a knot of a "bow-tie module" playing a role in the aging process (Fig. 6) (Conte et al, 2022).

Figure 6. GDF15 may be considered as a knot of a bow-tie architecture. The knot (GDF15) accepts several stresses and stimuli (input or fan in), and produces a wide variety of biological effects (beneficial or detrimental output or fan out). This module might also be equipped with a regulatory feedback loop, able of modulating the knot (*from Conte et al, 2022).*



The bow-tie model was introduced to describe complex biological systems characterized by several inputs signals (fan in) converging into a knot, where they are ordered, processed, and propagated as multiple outputs signals (fan out) (Csete and Doyle 2004; Tieri et al., 2010; Friedlander et al, 2015). This model could be applied to GDF15. In particular, especially during aging, many stress stimuli (fan in) can activate TFs that are able to enhance GDF15 expression. GDF15, in turn, has different biological effects, both central and peripheric on various tissues, as seen (fan out). Moreover, it is possible that the GDF15 bow-tie architecture is flexible and equipped with a feedback loop, able of modulating the knot, to convey different effects in response to different stimuli to which the organism may be exposed. This may reduce the number of central molecular mediators and the energy expenditure, that would otherwise be necessary to respond to the multitude of stimuli associated with aging and ARDs (Conte et al, 2022) (Fig. 6).

2.4 GDF15 in aging and ARDs

As already mentioned, GDF15 is one of the most upregulated circulating proteins during aging and is associated to several ARDs. Given the various biological effects that GDF15 has (as illustrated in the previous paragraph), it is not always easy to understand the specific role of GDF15 in the onset

or progression of ARDs and is not yet clear if it is protective or detrimental (Conte et al, 2022). Contrasting evidence has been reported regarding the association of GDF15 with different ARDs.

In physiological conditions, the level of circulating GDF15 is low and usually in a range between 200 and 1200 pg/ml (Kempf et al, 2007, Conte et al, 2020). Interestingly, GDF15 plasma level is markedly elevated at birth with a concentration of about 3000 pg/ml and declines to levels typical of health adults in the first 4 months of life (Díaz et al, 2020). In centenarians this level can reach a value of about 4000-5000 pg/ml or more (Conte et al, 2019). Many stressors, such as physical exercise, fasting and high fat diet can transiently increase the level of GDF15 (Conte et al, 2020; Klein et al, 2021). Moreover, the circulating level of GDF15 shows a diurnal variation of approximately 10%, not associated to meals or caloric consumption (Patel et al, 2019). The circulating level of GDF15 can dramatically increase in several ARDs and pathological conditions.

Consistently with its definition of **mitokine**, circulating GDF15 levels are elevated in animal models of mitochondrial dysfunction and in patients with mitochondrial diseases. Mitochondrial stress is considered a driver and a hallmark of aging, however, as mentioned before, a mild mitochondrial stress can be part of an adaptive stress response that promotes longevity and healthspan. This phenomenon is known as mitohormesis, and various authors have proposed that GDF15 could be a mediator of it. The exact role of GDF15 in ARDs, characterized by mitochondrial dysfunction, is not completely clear. It is possible that GDF15 acts as a link between aging, mitochondrial dysfunction and ARDs (Conte et al, 2022).

The plasma level of GDF15 is not only elevated in patients with different ARDs, but often seems to reflect the severity and progression of the disease. Therefore, GDF15 is now considered both a biomarker and a potential target of several ARDs. Moreover, GDF15 levels were found to correlate with lower survival in old age (Conte et al, 2019; Conte et al, 2022). On the contrary, one study performed in transgenic mice overexpressing GDF15 suggested that GDF15 could act as a survival factor promoting lifespan (Wang et al, 2014).

By way of example but not limited to, elevated GDF15 circulating levels have been associated with several types of cancer, cardiovascular diseases, cachexia, sarcopenia, type 2 diabetes, neurodegeneration, renal dysfunction and inflammatory diseases (Conte et al, 2022). In addition, recent studies found that high levels of plasma GDF15 were associated with COVID-19. In particular, GDF15 level was higher in hospitalized patients compared to controls, and non-survivors had higher GDF15 serum level after intensive care unit admission, compared to survivors (Notz et al, 2020; Myhre et al, 2020; Conte et al, 2022).

A detailed description of the association between GDF15 and every single ARD is outside the scope of this thesis, but a specific focus on the link of GDF15 with sarcopenia and neurodegenerative diseases, two types of ARDs considered in the present study (see Results section), will be given.

An association between GDF15 plasma levels and **sarcopenia** has been suggested. In particular, a study showed that circulating GDF15 could predict sarcopenia in patients with chronic obstructive pulmonary disease (Deng et al, 2022). Another work found that GDF15 could be useful as a predictor of sarcopenia in aged mice and humans (Kim et al, 2020). Other studies showed an association of GDF15 with muscle wasting, decreased muscle performance and increased inflammation (Conte et al, 2020; Alcazar et al, 2021). An *in vitro* study performed in C2C12 cells, found that the treatment with recombinant GDF15 led to increased expression levels of *MuRF1* and *Atrogin,* two atrophy-related genes, and to reduced expression of muscle-specific miRNAs (Bloch et al, 2015). In contrast with these studies, there is a paper suggesting that GDF15 could not predict the risk of sarcopenia. In fact, in this paper, the authors found that serum levels of GDF15 in sarcopenic patients were similar to those observed in non-sarcopenic controls (Nga et al, 2021). Even if several studies suggested an association of GDF15 with sarcopenia and muscle strength decline, the exact role of GDF15 in these phenomena is still unclear.

Regarding the link of GDF15 with **neurodegenerative diseases**, contrasting data emerge from scientific literature. A recent study, explored the association of serum GDF15 with cognitive performance. The authors found that GDF15 was associated with poorer scores in different cognitive tests, however, GDF15 level was not able to predict the rate of change in cognitive performance over time (Beydoun et al, 2023). A systematic review found that circulating levels of GDF15 were significantly higher in patients with neurodegenerative diseases with respect to healthy people (Xue et al, 2021). In another study, the authors performed a Mendelian randomization and the analysis showed a possible association of elevated GDF15 levels and the risk of Alzheimer's disease (**AD**) (Wu et al, 2021). Chai and colleagues found that higher circulating GDF15 levels were associated with dementia and cerebrovascular disease (Chai et al, 2016).

In contrast with these studies, other works found protective/beneficial roles for GDF15 in neurodegenerative diseases or did not find an association of higher levels of circulating GDF15 with higher risk of neurodegenerative disease. For example, in a 2021 study, we found that plasma GDF15 level was similar in patients with AD and healthy age-matched controls (Conte et al, 2021). Moreover, studies performed in *in vitro* and animal models found protective roles for GDF15 in brain health. For instance, a study showed that GDF15 KO in a mice model of Parkinson's disease affected dopaminergic neurons survival (Machado et al, 2016). Another study showed that the administration

of exogenous recombinant GDF15 in cultured microglial cells promoted the clearance of β-amyloid (Kim et al, 2018).

Considering the contrasting evidence briefly showed here, it is still not clear the exact role of GDF15 in aging and, thus, in promoting or contrasting ARDs. Our hypothesis is that GDF15 is part of an adaptive response, aimed to counteract the stress and thus is basically beneficial. In particular, the age-related increase in GDF15 levels is part of a response to various increasing stresses that occur during aging. However, during aging, stresses may become more frequent and unsolved, leading to a chronic upregulation of GDF15 which can in turn become detrimental. Accordingly, the association of GDF15 with pathologies is observed in conditions of chronically elevated levels of GDF15 (Conte et al, 2022). This hypothesis can also be integrated in the *continuum* hypothesis of aging. In fact, it could be suggested that the stresses that cause the chronic elevation of GDF15 level are the same driving aging and ARDs. Therefore, the apparent involvement of GDF15 in both processes may be possibly due to unwanted effects on metabolism and immune defences (*e.g.*, cachexia, immune depression) (Franceschi et al, 2018; Conte et al, 2022):

Finally, another possibility is that when the intensity of the stress is too high, GDF15 is not able to restore the homeostasis and the net result is the onset of an ARD. In this case the association observed between the level of GDF15 and a specific ARD should be considered indirect or even spurious (Conte et al, 2022).

2.5 Evolutionary and ecological perspectives of GDF15

GDF15 shows important structural differences, which set it apart from its counterparts in the TGF-β superfamily. Orthologues of GDF15 have been found in different species, and in particular in mammals, reptiles, amphibians, bony fish and birds. GDF15 likely evolved in the common ancestor of jawed vertebrates since there is no obvious orthologue in the other two lineages of craniata, hagfish, lampleys, and lower vertebrates. The C-terminal of the protein is highly conserved, whereas the propeptide conservation is lower, suggesting a strong remodeling and simplification during evolution. Amino acid alignment shows that there are important differences in the sequence of GDF15 of placental mammals and the other orthologues (Lockhart et al, 2020). Protein sequence data from UniProt shows that human GDF15 has 90% identity with Ma's night monkey, Northern white-cheeked gibbon, Pygmy chimpanzee, Silvery gibbon, Bonobo, and Chimpanzee, while no other species present 100% identity (Conte et al, 2022). The RXXR furine-like cleavage site, needed for protein maturation, as seen before, is present in human, mouse, rat, canine, and chimpanzee GDF15 (Baek and Eling, 2019). Moreover, it has been shown that species-specific expression of GDF15 may be driven by a human-specific sequence. In fact, alignment of 46 vertebrates showed that the genomic

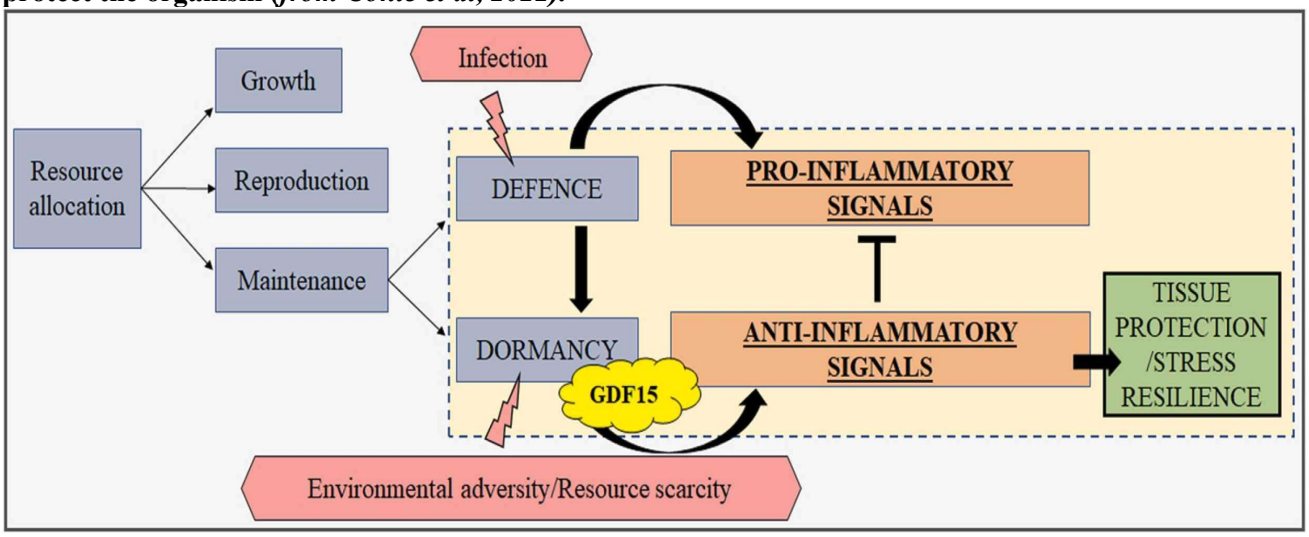
sequence of the enhancer near GDF15 is conserved across multiple vertebrates, but is absent in mouse genome (Ulirsch et al, 2014).

To date, evolutionary data on GDF15 suggest an increasing importance of GDF15 in primate evolution. An evolutionary study performed on primates identified a correlation between the rate of protein evolution of GDF15 and different life-history traits, such as maximum lifespan, weaning time, female age at maturity and gestation (Conte et al, 2022; Muntané et al, 2018).

The life-history theory is an evolutionary theory, used in the study of aging, that proposes that living organisms allocate limited resources into different main biological programs, that can be distinguished in: i) growth; ii) reproduction; iii) maintenance to maximize reproductive success (Stearns, 2000). This theory can explain different resources allocation in different environments and in response to different stimuli and stressors. In 2019, Wang and colleagues proposed a further subdivision of the maintenance program in two subfields: i) the **defense** program and ii) the **dormancy** program. In particular, the defense program is activated when an organism must face different factors, such as pathogens that cause infections. This program relies on the anabolic metabolism to invest energy in mounting protective responses and requires a great amount of energy. On the other side, the dormancy program is activated in presence of an adverse environment or absence of resources. This program relies on the catabolic metabolism and tends to preserve energy. Even if these two programs can work separately, inflammation can activate several components of the dormancy program. In fact, surviving an infection requires pathogens clearance but also tissue protection from both pathogens-caused damages and inflammation. This is achieved by a defense-induced dormancy program. (Wang et al, 2019; Conte et al, 2022). Different molecules play a role in the dormancy program. For example, the mitokine FGF21 is a pivotal signaling molecule that may promote tissue protection in case of an inflammatory state. **GDF15** shows similar biological effects and could be considered as a mediator of the dormancy program (Conte et al, 2022). In particular, GDF15 has been identified as a key factor for tissue tolerance to inflammation in both bacterial and viral infections, through the modulation of systemic levels of triglycerides (Luan et al, 2019). Other actions of GDF15 led to the idea that it could be a key player in the dormancy program. As briefly mentioned before (paragraph 2.3), different studies showed that GDF15 can protect cells from apoptosis, promoting cell survival (Abulizi et al, 2017, Heger et al, 2010). Moreover, GDF15 also promote fatty acids catabolism (Zhang et al, 2018) and the maintenance of body temperature in mice (Luan et al, 2019). Finally, recent data suggest a neuroprotective effect of GDF15, showing that it is able to prevent retinal ganglion cell loss after retinal nerve crash in mice (Iwata et al, 2021). All this evidence suggests a role of GDF15 as a potential key mediator of the dormancy program, able to

guarantee cell/organismal passive protection and resilience, thus highlighting its importance during the primate evolutionary process (Fig. 7) (Conte et al, 2022).

Figure 7. GDF15 is proposed as a mediator of the dormancy program, one of the two arms of the maintenance mechanisms, according to the life-history theory. The defense program can activate the dormancy and, conversely, the dormancy mechanisms can modulate the defense responses in order to protect the organism (*from Conte et al, 2022).*



Aim of the thesis

Aging is a complex and inevitable process and is the main risk factor for many, if not all, the aging-related diseases (ARDs). Biologically speaking, aging is characterized by twelve hallmarks and, according to the *continuum* hypothesis of aging, the majority of these hallmarks are sustained by mechanisms that are shared between aging and ARDs (López-Otín et al, 2023; Franceschi et al, 2018). Among these biological processes two are of particular interest and widely studied: inflammation and mitochondrial dysfunction. In particular, the low-grade chronic and sterile inflammation associated to aging is defined inflammaging. Inflammaging has also a counterpart known as "anti-inflammaging", whose mechanisms are still under investigation (Franceschi et al, 2000). Among the molecules identified as possible mediators of anti-inflammaging are mitokines and, in particular, GDF15.

GDF15 is a stress-response molecule, produced in response to a plethora of stresses and stimuli, but in particular in response to mitochondrial stress within the UPRmt. Interestingly, GDF15 is one of the most upregulated protein during aging and is closely associated with several ARDs (Tanaka et al, 2018; Conte et al, 2019; Conte et al, 2022). Although different biological actions of GDF15 have been identified or proposed, to date, the exact role of this protein in the aging process and in the development of ARDs is still unclear.

Several studies have associated increased levels of circulating GDF15 with different ARDs, including neurodegenerative diseases and sarcopenia. We found that GDF15 circulating level in patients with Alzheimer's disease (AD) was similar to that observed in age-matched non-demented subjects (Conte et al, 2021). Moreover, we and others showed that the level of circulating GDF15 was increased in patients with lower limb mobility impairment (LLMI) and decreased muscle strength and was associated to inflammation (Conte et al, 2020). However, the majority of the studies performed to investigate the link between GDF15 and ARDs are focused on the circulating level of this protein, and little is known about the intra-tissue level of GDF15 in specific physiological and/or pathological conditions.

The aim of this thesis was therefore to analyze the tissue level of GDF15 in two prototypes of common ARDs, namely in the brain of patients who were affected by AD and in the skeletal muscle of patients with LLMI. Patients and healthy controls were also subdivided in different age-groups, which allowed us to analyze GDF15 tissue levels (and other parameters related to metabolism, inflammation and mitochondria) also from an aging-related perspective.

Moreover, to better clarify the role and molecular pathways that may involve GDF15 in aging and ARDs, we took advantage of primary cultures of human dermal fibroblasts, obtained from subjects

of different age (young and old) and from patients with AD. GDF15 knock-down and comparisons between the behavior of cells from healthy subjects and AD patients were performed.

Materials and methods

1 Human samples

1.1 Cerebrospinal fluid (CSF) and brain samples (*study on Alzheimer's disease)*

Cerebrospinal fluid (CSF) samples from 48 subjects in the age range 54-81 years were used. The subjects were admitted to the Alzheimer's Center of the University of Milan, Fondazione IRCCS Ca' Granda, Ospedale Policlinico, with suspicion of neurodegenerative dementias. The clinical workup included detailed past medical history, general and neurological examination, routine blood tests, formal neurocognitive assessment, brain computed tomography (CT) scan or magnetic resonance imaging (MRI), and, when indicated, [18F]-fludeoxyglucose positron emission tomography, as well as lumbar puncture (LP) for CSF biomarkers amyloid beta (Aβ-42), total Tau (T-Tau), and tau phosphorylated at position 181 (P-Tau181) determination. Normality references considered were Aβ-42 ≥ 600 pg/ml; T-tau ≤ 500 pg/ml for individuals older than 70 years and ≤ 450 pg/ml for individuals aged between 50 and 70 years; and P-tau 181 ≤ 61 pg/ml. The diagnosis of AD was done according to current criteria (Dubois et al, 2014). In order to compare the data obtained from CSF with data obtained from plasma, as described in Conte et al, 2021, AD patients were selected from our previous study for plasma GDF15 levels (Conte et al, 2021).

The study was approved by the local ethics committee (study n. 5,802 approved on 14-09-2021 by Comitato Etico Milano Area 2).

CSF samples were divided in: i) 8 subjects diagnosed as non-AD with low levels of CSF T-Tau (<350 pg/ml) and mild cognitive impairment stable over at least 1-3 years of follow-up (mean age 73±7.1 years); ii) 20 AD patients with low CSF T-Tau level (<400 pg/ml) (mean age 69.9±8.4 years); iii) 20 AD patients with high CSF T-Tau level (>400 pg/ml) (mean age 70.2±8.4 years). The subjects diagnosed as non-AD were not cognitively impaired according to the mini-mental state examination (MMSE) test (MMSE> 28). Moreover, the non-AD subjects did not present alterations with instrumental analyses (CSF biomarkers and imaging) and did not develop dementia over a 1–3 year follow-up.

CSF samples were collected into 15ml polypropylene tubes by LP in the L3/L4 or L4/L5 interspace in the morning, after overnight fasting. After LP, CSF samples were centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatants were aliquoted and stored at -80°C until use. For each CSF sample Aβ-42, T-tau, and P-tau181 were measured using, respectively, three commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits: INNOTEST Amyloid-beta 42, tau, and P-tau181 assays (INNOTEST Fujirebio, Ghent, Belgium), conducted according to the instructions of the manufacturer.

Human autopsies of different brain areas from 32 subjects in the age range 33-104 years were also used. The samples were provided by two different biobanks: 1) Abbiategrasso Brain Bank at the Golgi Cenci Foundation (Milan, Italy) and 2) the immunology lab at Bologna University (responsible: Prof. S. Salvioli).

1. The 19 samples from the Abbiategrasso Brain Bank were divided into i) 6 non-demented old control subjects (NDO, age range 79-80 years) without clear signs of neurodegeneration; ii) 12 patients with Alzheimer's disease (AD) (age range 75-89 years); iii) 1 centenarian subjects with age-related Tau-pathology (104 years). The study protocol was approved from the Ethical Committee of Pavia University (Committee report 3/2009).
2. The 13 samples from the immunology lab at Bologna University were divided into: i) 10 non-demented subjects [3 adults in the age range 33-55 years (NDA) and 7 old in the age range 71-82 years (NDO), without evident signs of neurodegeneration]; ii) 1 patient with a diagnosis of AD (69 years); iii) 2 centenarians affected by cognitive impairment who died of old age (103 years). All samples were collected in the framework of the European Project PROTEOMAGE (grant agreement: FP6-818230).

   The mean postmortem interval (PMI) for all subjects was 9.9 ± 4.5 h (range: 3–16 h).

ND subjects all died from pathologies not affecting the brain, and older ones displayed no signs of cognitive impairment at death; they presented only mild age-related neuropathological alterations. The samples categorized as AD were from patients with a clinical diagnosis of major neurocognitive disorder (major-NCD) according to DSM-5, and a neuropathological diagnosis of AD. The post-mortem diagnosis of AD was made according to the NIA-Alzheimer's association guidelines for the neuropathological assessment of AD, using the ABC score (Montine et al, 2012). A neuropathological analysis of the samples was also carried out on formalin-fixed slices, embedded in paraffin. The slices were stained with hematoxylin and eosin, cresyl violet, luxol fast blue, and Gallyas to evaluate vascular, architectural and structural tissue abnormalities, myelin loss, and neuritic plaques. For immunohistochemical analysis, NeuN and GFAP were used to evaluate neuronal and glial compartments, while AT8, 4G8, α-synuclein, and TDP43 antibodies were used to assess all the main proteinopathies.

1.2 Plasma and skeletal muscle samples (*study on lower limb mobility impairment)*

The subjects were recruited in the framework of the EU Project MYOAGE. The study protocol was approved by the Ethical Committee of Istituto Ortopedico Rizzoli, Bologna, Italy (ethical clearance no. 10823 on April 26, 2010). All subjects signed an informed consent before entering the study.

Plasma samples were obtained from two groups of subjects: i) 47 healthy subjects (HS) (mean age 57.1 ± 3.6 years) divided in 15 subjects <40 years of age (mean age 22.7±0.63) and 32 subjects >70 years of age (mean age 74.3±0.62); ii) 46 patients with lower limb mobility impairment (LLMI) (mean age 61.3±3.6 years) divided in 21 subjects <40 years of age (mean age 36.3±1.5) and 25 subjects >70 years of age (mean age 82.2±1.6). To ensure the selection of subjects in healthy conditions, some exclusion criteria were used: presence of comorbidities, inability to walk a distance of 250m, use of medications, immobilization for 1 week during the last 3 months and orthopedic surgery in the last 2 years. For the patient's group the exclusion criteria were: the presence of chronic kidney or liver diseases, bleeding disorders, severe diabetes mellitus, rheumatic diseases other than osteoarthritis, neuromuscular disorders, malignancies and systemic infections, chronic steroid use, major psychological problems or history of alcohol or drug abuse, evidence of prior surgery in the involved hip.

Blood samples were collected in the morning after overnight fasting. Plasma samples were obtained after a 15 minutes centrifugation at 2000g at 4°C. Samples were then rapidly frozen and stored at -80°C until the analyses were performed.

Muscle biopsies were also used for this study. Biopsies were taken from the *vastus lateralis* (VL) of 23 HS, after localized anesthesia, and from 16 LLMI, during the operation at the site of surgical incision. All biopsies were immediately frozen in liquid nitrogen and then stored at -80°C.

2 Cell cultures, *in vitro* gene KD and doxorubicin treatment

Dermal fibroblasts (DFs) were obtained from biopsies of sun-protected areas from 11 non-demented subjects without neurodegenerative diseases (3 young subjects in the age range 25–34 years and 8 old subjects in the age range 73–78 years) and from 3 AD patients (age range 75–79 years). DFs from control subjects were from Salvioli's Lab in Bologna University, whereas those from AD patients were from the Abbiategrasso Bank at the Golgi Cenci Foundation. In these latter subjects, the biopsy was performed at an average time of approximately 8 h post-mortem. Cells were cultured in DMEM-high glucose supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 mg/ml), and 2 mM L-glutamine (all from Sigma), in an incubator at 5% CO_2, with a humidified atmosphere of 37°C. All the DFs used for the experiments were between the 5th and 12th passages.

K562 cells (an immortalized myelogenous leukemia cell line) were available at the Salvioli's lab, OV90 cells (a cell line derived from the ovary of a patient with malignant papillary serous adenocarcinoma) were kindly provided by Prof. Iommarini and Porcelli (University of Bologna).

These cells were cultured in DMEM supplemented as previously described for DFs and maintained in an incubator at 5% CO_2, with a humidified atmosphere of 37°C.

GDF15 KD was obtained through an RNA interference (RNAi) strategy. siRNA targeting human GDF15 and scramble negative control siRNA were provided by Cohesion Biosciences. A specific combination of GDF15 siRNA was selected after testing the silencing efficacy of different combinations of three different siRNA targeting GDF15, for each different cell line (fibroblasts, K562 and OV90). Only for DFs, Perilipin 2 (PLIN2) KD was also performed, with the same technique used for GDF15 KD. Transfection was performed with ScreenFect siRNA reagent (ScreenFect GmbH), following the manufacturer's instructions. For DFs, 125000 cells were seeded in a 6-well plate and scramble negative control siRNA, GDF15 or PLIN2 siRNA was added to the cells with the transfection reagent. The medium with the siRNA was replaced after 24 h with a fresh complete medium. Cells were then harvested after a further 72 h for RNA extraction. The KD in K562 and OV90 cells was performed in 24-well plates, by reseeding 40000 cells/well. Medium with the siRNA was replaced after 24h and cells were harvested for further analyses after a further 48h.

Doxorubicin treatment was performed in 12-well plates. Cells were reseeded (62500 cells/well for DFs; 80000 cells/well for K562 and OV90) and treated for 24h with different doses of doxorubicin. After this time, cells were harvested for RNA extraction.

3 Measurements on plasma, CSF and cell supernatant

Plasma IGF-1, Insulin, Adiponectin, Leptin, Resistin, IL6 and GDF15 concentrations were obtained using commercial ELISA kits (Quantikine R&D Systems), according to manufacturer's instructions. Circulating Perilipin 2 (c-PLIN2) was measured using the ELISA commercial kit Human ADRP ELISA (E-EL-H0278, Elabscience), according to the manufacturer's instructions. Each analyte was measured in duplicate for each sample. Plasma IL15 was analyzed using the Simple Plex Human IL-15 Cartridge (ProteinSimple/Bio-Techne) run on an Ella Automated Immunoassay System (ProteinSimple/Bio-Techne), according to manufacturer's instructions.

Quantikine ELISA Human GDF15 kit was also used to quantify GDF15 in CSF and in cell supernatant.

Synergy™ fluorometer (Bio-Tek Instruments, Winooski, VT, United States) was used to read the absorbance of the plates.

4 RNA extraction and gene expression analysis

As regard brain samples, RNA was available for frontal cortex samples only. Total RNA was isolated from about 50mg of tissue using the RNeasy Lipid Tissue Mini kit (Qiagen). The tissue was

homogenized with the OMNI TH Tissue Homogenizer (OMNI international) in the lysis buffer supplied by the kit. RNA isolation was then performed following the manufacturer's instructions. RNA concentration and purity were checked on a NanoDrop2000 spectrophotometer (Thermo Scientific), whereas RNA integrity was analyzed using a 2100 Bioanalyzer (Agilent Technologies). Samples with an RNA integrity number (RIN) ≥ 4 were included in the gene expression analysis.

Total RNA from DFs, K562 and OV90 was isolated from cell pellets using the EasyPure RNA kit (TransGen Biotech Co., Ltd) according to the manufacturer's instructions.

cDNA was synthetized using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. Gene expression was analyzed by real-time RT-PCR, performed with iTaq™ Universal Sybr Green Supermix (Bio-Rad) and a Rotor gene Q 6000 system (Qiagen). Different housekeeping genes were tested for their stability as reference genes (*RNA18S1*, *ACTB*, ribosomal protein large P0, phospho-glycerate kinase 1 [*PGK1*], and glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]). *PGK1* (brain samples) and *GAPDH* (cell culture samples) were chosen as reference genes due to their more stable results and all data were normalized with respect to these genes. The relative expression ratio was then calculated using the $2^{-\Delta\Delta CT}$ method. All the primers used in this study were pre-designed and pre-validated and purchased from Bio-Rad. More information about primers is available at www.bio-rad.com/PrimePCR.

5 Protein extraction and Western Blotting analysis

As regard brain autopsies, protein lysates were obtained from about 50mg of frozen samples of different brain areas, with the exception of centenarians from which only frontal cortex samples were available. A lysis buffer with the following composition was used: CHAPS 4%, Urea 8 M, DTT 65 mM, Tris 40 mM, phosphatase, and protease inhibitors (Sigma).

As regard skeletal muscle biopsies, proteins were obtained from about 40mg of frozen tissue, using the TEAD buffer (Tris-HCl 20 mM pH= 7.5, EDTA 1mM, NaN3 1mM, DTT 1mM) with protease and phosphatase inhibitors (Sigma).

For both tissues, lysis was performed with the OMNI TH Tissue Homogenizer (OMNI international). The lysates were then centrifuged at 25000 rpm for 1 h at 4°C and the supernatant was collected.

All protein extracts were quantified by Bradford's method and stored at −80°C until the analysis.

50µg (brain samples) or 10µg (muscle samples) of total protein extract were separated on 12%, 16% or 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) polyacrylamide gel. Proteins were then transferred to a Polyvinylidene Difluoride (PVDF) or nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) and immunoblotted with the adequate primary antibodies (Table 1).

Table 1. List of primary antibodies used for western blotting experiments.

Primary antibody	Supplier	Dilution used
Gapdh	Novus Biological	1:20000
Gdf15/Mic-1	Cell Signaling	1:500
VDAC1	Abcam	1:2000
Cox IV	Proteintech	1:1000
NDUFA9	Abcam	1:1000
SDHA	Abcam	1:2500
UQCRC2	Abcam	1:1000
ATP5H	Abcam	1:1000

GAPDH was used as housekeeping for normalization. Images acquisition was performed with ChemiDoc Imaging System (Bio-Rad). Band densitometry analysis was performed with Fiji software.

6 Immunofluorescence

The co-localization of different antigens such as Neuronal Nuclei (NeuN) and GDF15, TMEM119 and GDF15, as well as GDF15 and Glial Fibrillary Acidic Protein (GFAP), was investigated in control and AD brain tissue sections, using a double sequential immunofluorescence procedure. Formalin-fixed paraffin-embedded sections were deparaffinized in xylene, rehydrated in ethanol at decreasing concentrations, and washed in distilled water. Then, tissue sections were pre-treated using microwave (4 cycles for 5 min each) and citrate buffer pH 6.0 for antigens retrieval, cooled to room temperature (RT) for 15 min, and rinsed in PBS for 10 min. Afterward, slides were incubated with 1% bovine serum albumin (BSA) in PBS at RT for 30 min to block unspecific binding sites, probed with monoclonal NeuN (1:1,000; Chemicon, Millipore) or TMEM119 (1:500; Novus Biologicals) and polyclonal GDF15 (1:100; Cohesion Biosciences) primary antibodies and incubated at 4°C overnight; polyclonal GFAP (1:500; Dako), was incubated at RT for 30 min. Nuclei were counterstained with ProLong™ Gold antifade reagent with DAPI (Invitrogen by Thermo Fisher Scientific) and slides were stored at 4°C. Negative controls were obtained by processing sections without primary antibodies. Digital images were acquired using a Leica DMI6000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

7 Transmission electron microscopy (TEM)

For ultrastructural analysis of DFs, cells were seeded in a 6-well plate (125000 cells/well) and scramble siRNA or GDF15 siRNA were added to the culture medium. After 96h, DFs were washed

in PBS before being fixed in 2.5% buffered glutaraldehyde at RT for 20 min. From each well, cells were recovered by scraping and then transferred into microtubes before proceeding with centrifugation. The obtained cellular pellets were stored in the same fixative at 4°C overnight. Then, samples were rinsed, post-fixed in 1% buffered osmium tetroxide for 1 h at RT, gradually dehydrated with ethanol through increasing concentrations, and embedded in Araldite resin. After sectioning samples with ultramicrotome, the ultrathin sections were counterstained with uranyl acetate and lead citrate and observed in a Philips CM100 (FEI Company, ThermoFisher, Waltham, MA, United States) Transmission Electron Microscope, and digital images were acquired with an Olympus camera.

8 Muscle strength and body composition measurements

For HS, isometric quadriceps strength (IQS) was measured with a quadriceps chair. The subjects were positioned in an upright position, with straps to fix the hips to the chair and the ankle to the force transducer, at the knee angle of 90 degrees. Three trials were conducted to measure maximal voluntary contraction of the quadriceps. Each trial was separated by one minute of rest. The trial with the highest force output was taken for analyses.

For LLMI, the IQS was measured in seated position using a Handifor dynamometer. After a warming up period, patients were asked to perform three series of 10 contractions, progressively increasing the strength developed. The highest peak torque was withheld for analyses.

Only for LLMI, ultrasound imaging of the VL was performed using a portable ultrasound (Mylab25, Esaote) with a 7–10 MHz linear probe. Acquisition was performed by a trained examiner. Muscle thickness was calculated as the vertical distance between muscle superficial and deep aponeuroses, at an equidistant point from right and left borders of the sagittal image

Only for HS, a whole-body scan to detect total fat and lean mass was performed. The scan was performed using Dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500, version 12.4, Hologic Inc., Bedford), by a trained technician.

9 Analysis of cell senescence

Cell senescence was evaluated histochemically in DFs with the Cell Senescence Detection Kit (Abcam) according to manufacturer's instructions. Briefly, DFs cultured in 6-well plates were fixed with the fixative solution provided by the kit and then stained with the staining solution mix that allowed to detect the β-galactosidase activity. After an overnight incubation at 37°C, cells were observed using an inverted microscope. Semiquantitative analysis of cell senescence was performed using Fiji software. At least five images were analyzed per each sample and the experiment was

performed on three biological replicates for each age group. The percentage of β-galactosidase-positive cells out of the total number of cells was considered.

10 Mitochondrial oxygen consumption rate measurement

Mitochondrial oxygen consumption rate (OCR) was measured in DFs using the protocol described for Sea Horse XFe Mito Stress Test Kit (Agilent). 2750 cells/well were seeded in 100 µL of DMEM medium into XFe 96-cell culture plates and incubated at 37°C and 5% CO2. Upon KD of PLIN2, GDF15 or GDF15+PLIN2, complete growth medium was replaced with 180 µL of unbuffered XF medium (Agilent) supplemented with 10 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, at pH 7.4. After three OCR baseline measurements, 1 µM oligomycin (O), 1.5 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 1 µM rotenone (R), and 1 µM antimycin A (A) were sequentially added to each well. FCCP concentration was optimized on DFs by titration before the experiments. After each injection three measurements over time were performed, indicated as timepoints. Following the manufacturer's protocol, basal OCR was calculated using the values at Timepoint 3 subtracted from the non-mitochondrial OCR, namely values obtained at Timepoint 15, after antimycin A injection for which a complete block of the mitochondrial respiratory chain is achieved by Complex III inhibition. ATP-linked OCR was calculated subtracting OCR values at Timepoint 3 from OCR values at Timepoint 4 after injection of the specific F1Fo ATP synthase inhibitor oligomycin. Respiration associated with proton leak was determined as OCR values after oligomycin injection (Timepoint 4) subtracted for the non-mitochondrial OCR (Timepoint 15). Maximal OCR corresponded to the maximal value of respiration after FCCP injection subtracted for non-mitochondrial OCR. At the end of the assay, medium was removed and sulforhodamine B (SRB) assay was performed to determine protein content. Briefly, plates were incubated with 10% trichloroacetic acid (TCA) for 1 h at 4° C to fix the cells. Then, cells were washed five times with water. Completely dried plates were incubated with 0.4% SRB for 30 min at room temperature. Then, SRB was solubilized with 10 mM Tris and absorbance at 560 nm was determined using a Victor2 plate reader (Perkin-Elmer). Each biological replicate experiment ($n \geq 4$) included measurements from at least six wells. Data (pmol/min) were normalized to blank corrected SRB absorbance.

11 RNA sequencing analysis

Genome-wide transcriptome libraries were constructed on RNA extracted from DFs from young and old subjects transfected with GDF15 or scramble siRNA. The libraries were prepared with 150 ng-1

μg of total RNA using Illumina stranded mRNA prep ligation kit (Illumina) and dual indexes. Briefly, the purified and fragmented mRNA was converted to cDNA and amplified using Illumina primers containing dual indexes for each sample. Each library was subjected to quality control and quantification on Agilent Bioanalyzer using Agilent High Sensitivity DNA kit. An equal amount of libraries was pooled together and the library pool was re-quantified with Agilent High Sensitivity DNA kit and sequenced on Illumina NextSeq500 platform, using NextSeq 500/550 High Output Kit v2.5 (Illumina).

Raw base-call data generated from the Illumina NextSeq500 system were demultiplexed by Illumina BaseSpace Sequence Hub (https://basespace.illumina.com/home/index) and converted to FASTQ format. Then, the adapter sequences were trimmed using Cutadapt and reads were aligned to human reference genome hg38 using STAR tool. To reconstruct the RNA-Seq transcripts and quantify the expression, StringTie (v.2.1.7) was used. Differential expression analysis was performed in R environment (v.4.1.2) using Bioconductor's DESeq2 package. In order to identify differential expression, linear model testing that logFC differed from 0 was generated and obtained *p* values were adjusted for multiple testing with Benjamini-Hochberg (BH) algorithm. Genes with nominal *p* value <0.05 and absolute value or fold change after binary logarithm transformation (|logFC|) >1 were considered as significant.

12 Statistical analysis

Results are shown as mean ± SD or SE. All tests used are reported in each figure legend. After a Shapiro-Wilk normality test, Student's t test, ANOVA and Pearson correlation were used for normally distributed data whereas Mann-Whitney or Kruskal–Wallis tests and Spearman's correlation were used for data that did not follow a normal distribution. Bonferroni correction was applied to correct multiple comparisons. SPSS 17.0 for Windows was used for analyses. P values <0.05 were considered statistically significant.

To understand the role of circulating GDF15 (c-GDF15) and intramuscular GDF15 (SM-GDF15) in relation to other parameters (Insulin, Leptin, c-PLIN2, IL6, IL15, Resistin, Adiponectin and BMI) involved in muscle strength and mass loss, a principal component analysis (PCA) was used. Subsequently, a Canonical Discriminant Analysis (CDA) was used to evaluate which of the variables involved in the PCA were able to best discriminate the 4 groups of subjects involved in the test (healthy young subjects, young patients, healthy old subjects and old patients). The CDA is a dimension-reduction technique, related to principal component analysis and canonical correlation, able to perform both univariate and multivariate 1-way analyses. Given a classification character and

several interval variables, CDA derives a set of new variables, called canonical functions (CAN), which are linear combinations of the original interval variables.

Moreover, receiver-operating characteristics (ROC) curves were constructed to assess the discriminatory ability of the above-mentioned parameters in HS and LLMI.

Results

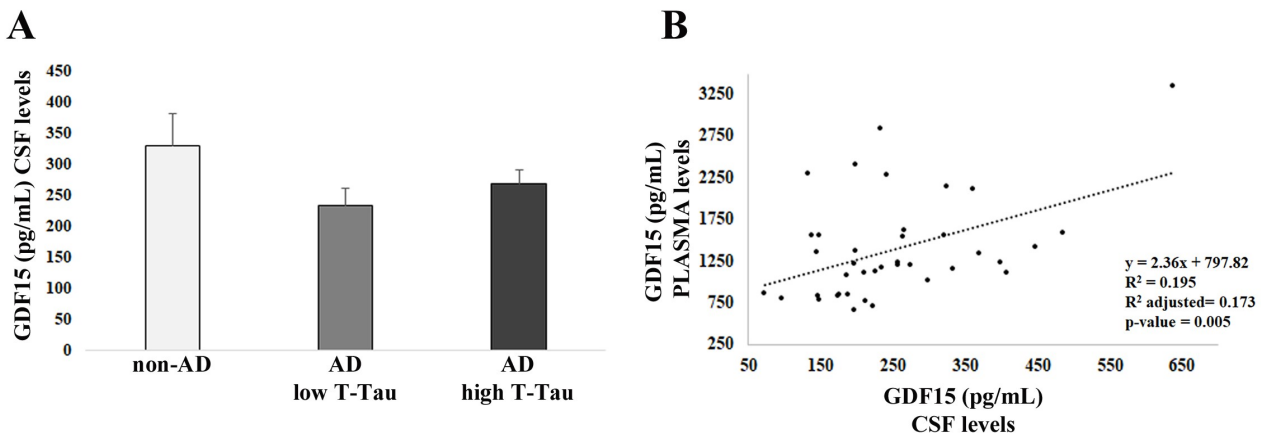
1 Characterization of GDF15 expression in brain from non-demented controls and patients with Alzheimer's disease (AD)

1.1 The CSF level of GDF15 is similar in AD patients and non-AD subjects

As mentioned before, in a previous work we found that GDF15 plasma levels were similar in AD patients and age-matched healthy controls (Conte et al, 2021). We then wondered if differences in GDF15 level were present in the CSF of AD patients, with either high or low levels of total Tau (T-Tau), considered a marker of the degree of neuronal damage, compared to age-matched non-AD subjects. We performed an ELISA test on the CSF of 40 AD patients and 8 non-AD subjects with mild cognitive impairment (MCI) stable over at least 3 years. No significant differences were found between the three groups of subjects (Fig. 8A).

We also performed a Spearman's correlation and regression analysis on GDF15 CSF level and GDF15 plasma level of the same subjects (as measured in Conte et al, 2021), that showed a positive correlation ($\rho = 0.378$; $p = 0.02$) (Fig 8B), suggesting a close relationship between the levels of GDF15 in the two biological fluids.

Figure 8. ELISA analysis of GDF15 level in CSF and plasma. (A) CSF level of GDF15 in 8 non-AD subjects, 20 AD patients with low T-Tau CSF level and 20 AD patients with high T-Tau level. (B) Regression analysis of GDF15 levels in the CSF and plasma of the same subjects.



1.2 *Gdf15* is expressed in human brain and its expression positively correlates with *Tp53, Atf3 and Il6.*

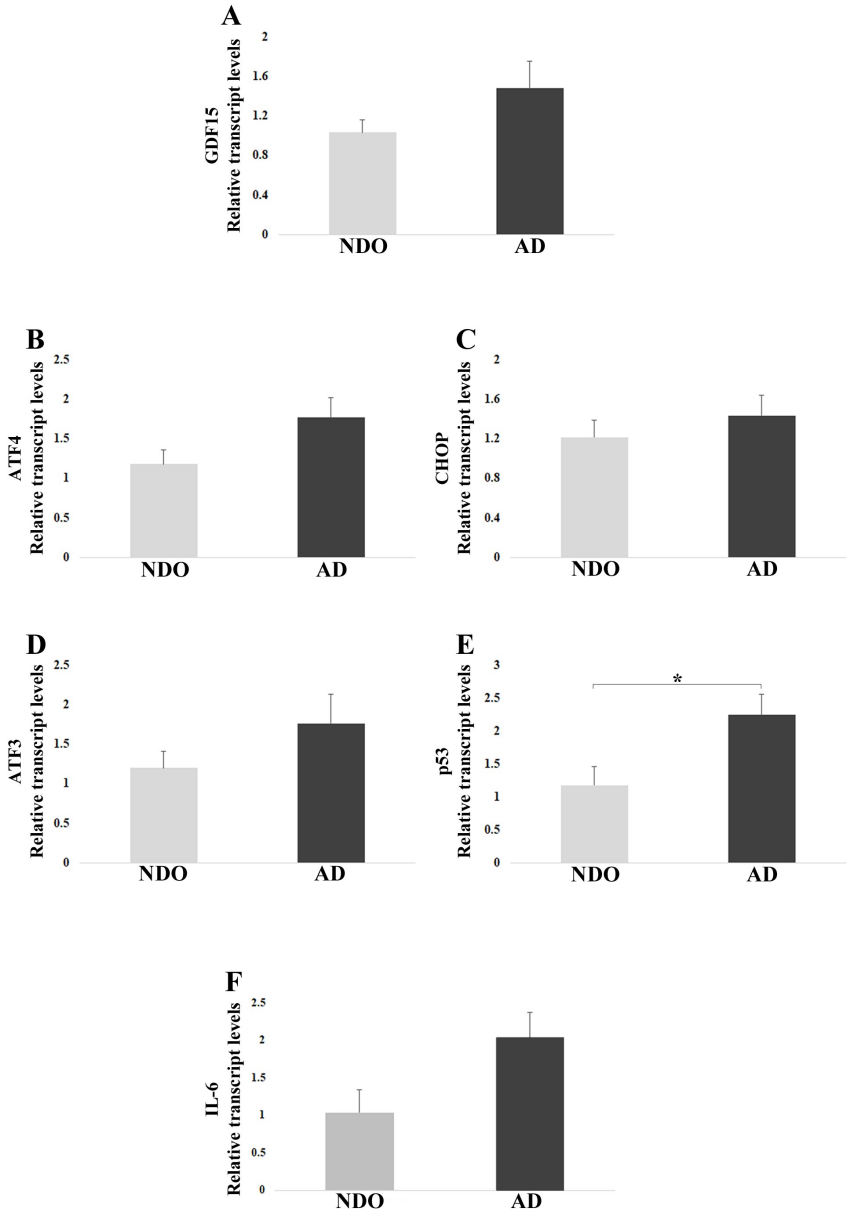
Even if we did not find differences between the level of GDF15 in the CSF of AD and non-AD subjects, we wanted to check if GDF15 could be expressed in brain tissue. Therefore, we performed a real time RT-PCR analysis in frontal cortex samples from 10 AD and 5 non-demented old subjects

(NDO). The transcript of *Gdf15* was detectable in both groups and the expression appeared to be higher in AD compared to NDO (Fig. 9A).

In order to obtain a more detailed characterization of the samples, we then analyzed the transcript level of some of the main transcription factors of GDF15: *Tp53, Atf3, DDIT3 (Chop)* and *Atf4.* The expression of all these transcription factors followed the same trend of *Gdf15*, with a higher expression in AD samples. However, only *Tp53* reached statistical significance (Fig. 9B-E).

We also analyzed the level of *Il6*, a pro-inflammatory cytokine strongly involved in AD and related to neuroinflammation. The level of *Il6* was higher in the frontal cortex of AD patients compared to NDO (Fig. 9F).

Figure 9. Real Time RT-PCR analysis of (A) GDF15, (B) ATF4, (C) CHOP (*DDIT3),* (D) ATF3, (E) p53 (*Tp53)* and (F) IL6 in 10 AD and 5 NDO frontal cortex samples.



We also checked for possible associations between the transcript levels of the analyzed genes, by performing a Pearson correlation analysis. A positive correlation of *Gdf15* with *Tp53*, *Atf3*, and *Il6* expression was found, as well as among *Il6*, *Tp53*, and *Atf3* transcripts (Table 2). Moreover, *p53* transcript levels positively correlated with *Atf3* and *DDIT3 (Chop)* (Table 2).

Table 2. Pearson correlation analysis of the transcript levels of the analyzed genes in frontal cortex samples from 5 NDO and 10 AD. (r: Pearson correlation coefficient; p: statistical significance).

		GDF15	IL-6	p53	ATF3	ATF4	CHOP
GDF15	r	1					
	p						
IL-6	r	0.680**	1				
	p	0.005					
p53	r	0.728**	0.645*	1			
	p	0.003	0.013				
ATF3	r	0.614*	0.605*	0.542*	1		
	p	0.015	0.017	0.045			
ATF4	r	0.061	0.096	0.359	0.229	1	
	p	0.830	0.734	0.208	0.412		
CHOP	r	0.389	0.076	0.698**	0.387	0.477	1
	p	0.152	0.788	0.006	0.154	0.072	

*p<0.05; **p<0.01

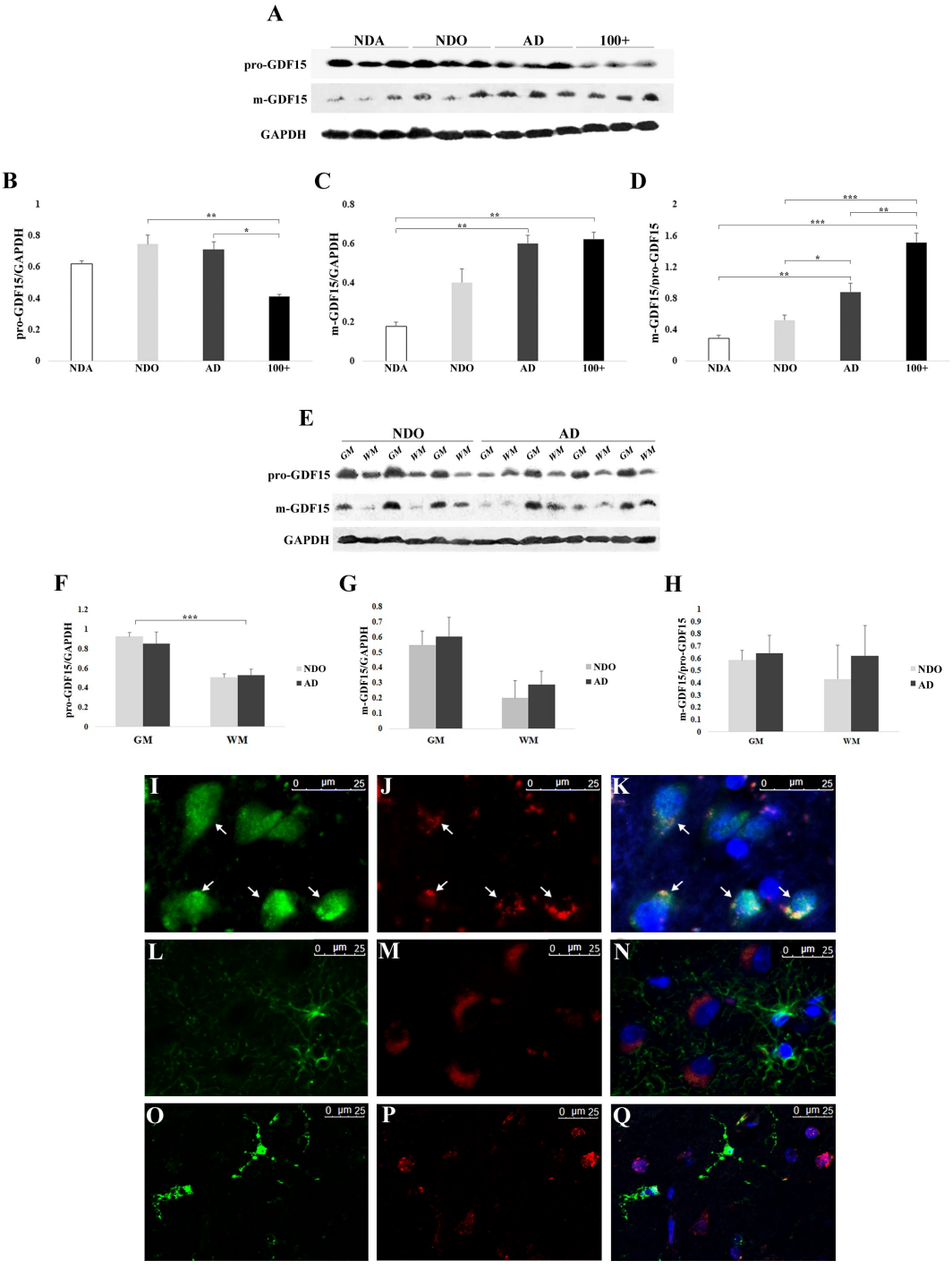
1.3 GDF15 protein is processed more in AD and centenarians' brain and appears to be predominantly expressed in neuronal cells

We then extended our analysis by evaluating the protein expression of the precursor (pro-GDF15) and the cleaved mature form (m-GDF15) of GDF15 in frontal cortex samples, not only from NDO and AD, but also from non-demented adults (age range 33-55 years) (NDA) and centenarians (100+). Pro-GDF15 expression was significantly higher in NDO and AD compared to 100+ (Fig. 10A, B). The expression of m-GDF15 was significantly higher in AD and 100+ as compared to NDA (Fig. 10 A, C). We then calculated the ratio between m-GDF15 and pro-GDF15, in order to have an indication of the rate of GDF15 processing. M-GDF15/pro-GDF15 ratio was significantly higher in AD and 100+ compared to NDA and NDO and also in 100+ compared to AD (Fig. 10D).

To have a further characterization of GDF15 expression in the brain, we took advantage of gray and white matter frontal cortex samples, from the same NDO and AD subjects. Both pro-GDF15 and m-GDF15 were expressed at higher levels in gray matter with respect to white matter (Fig. 10E-H).

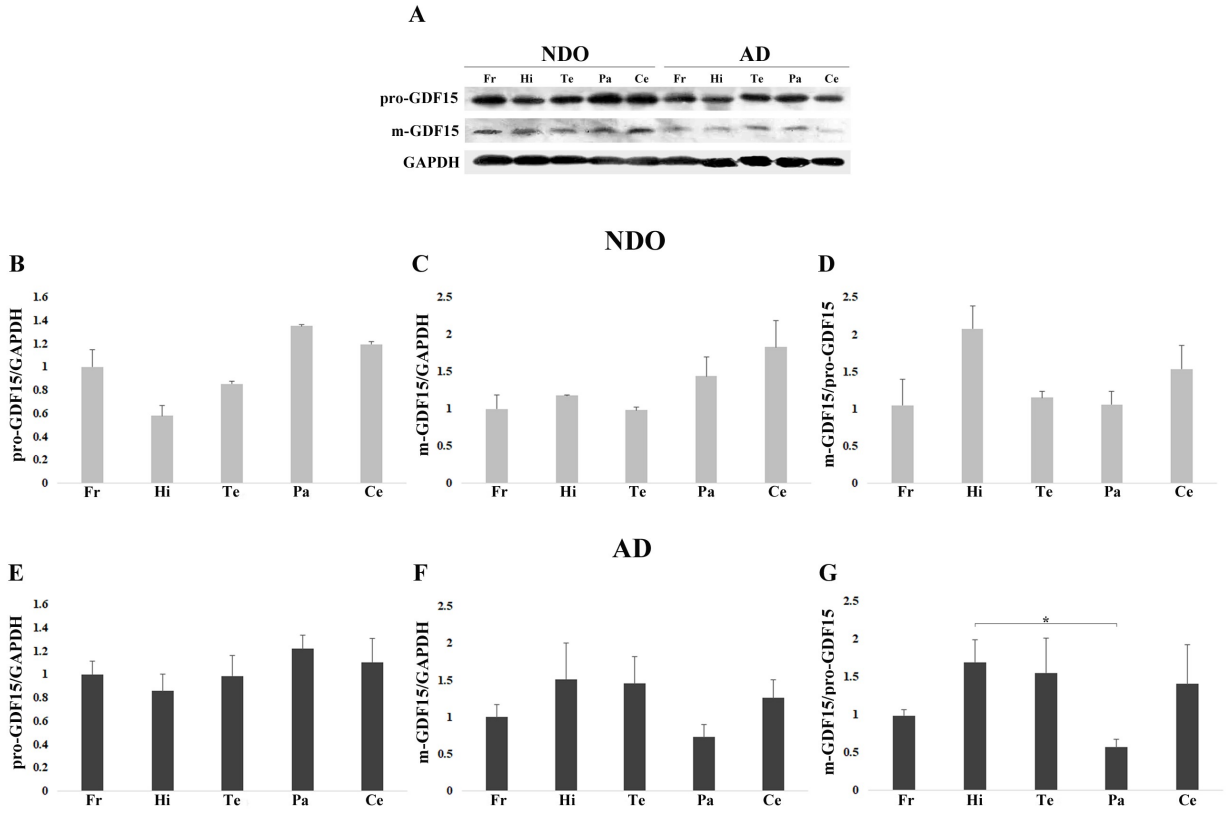
In order to assess whether GDF15 was expressed by all or only by specific brain cell types, we performed immunofluorescence analyses, in frontal cortex samples. In particular, we performed double labeling with specific antibodies for neurons (NeuN), astrocytes (GFAP), microglia (TMEM119) markers, and GDF15. The immunofluorescence analysis showed that GDF15 seems to be predominantly expressed by neuronal (NeuN+) cells, but not GFAP- nor TMEM119-positive ones (Fig. 10 I-Q).

Figure 10. Western blotting and immunofluorescence analyses of GDF15 in frontal cortex. (A) Representative immunoblotting image of pro-GDF15, m-GDF15 and GAPDH. Relative protein expression of (B) pro-GDF15, (C) m-GDF15 and (D) m-GDF15/pro-GDF15 ratio in 3 non-demented adults (NDA), 11 non-demented olds (NDO), 11 AD patients (AD) and 3 centenarians (100+). (E) Immunoblotting image of pro-GDF15, m-GDF15 and GAPDH in gray (GM) and white (WM) matter of frontal cortex. Relative protein expression of (F) pro-GDF15, (G) m-GDF15 and (H) m-GDF15/pro-GDF15 ratio in GM and WM from 3 NDO and 4 AD. (I-Q) Fluorescence microscopy analysis in frontal cortex. (I) NeuN (green), (J) GDF15 (red), and (K) merge; nuclei (DAPI). Arrows indicate colocalization of GDF15 and NeuN signals. (L) GFAP (green), (M) GDF15 (red), and (N) merge; nuclei (DAPI). (O) TMEM119 (green), (P) GDF15 (red), and (Q) merge; nuclei (DAPI). The bars represent mean ± SE. Student's t and one-way ANOVA tests with Bonferroni correction were applied. Western blotting quantification was performed using ImageJ software and normalized to GAPDH expression. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.



Since samples from other brain areas were available, we extended our analysis to check for possible differences in pro-GDF15 and m-GDF15 expression in different brain areas from NDO and AD. First, we compared pro- and m-GDF15 expression levels in frontal cortex, hippocampus, temporal cortex, parietal cortex and cerebellum. The levels of the two forms of GDF15 appeared largely similar in all brain areas analyzed, in both NDO and AD (Fig. 11A-G).

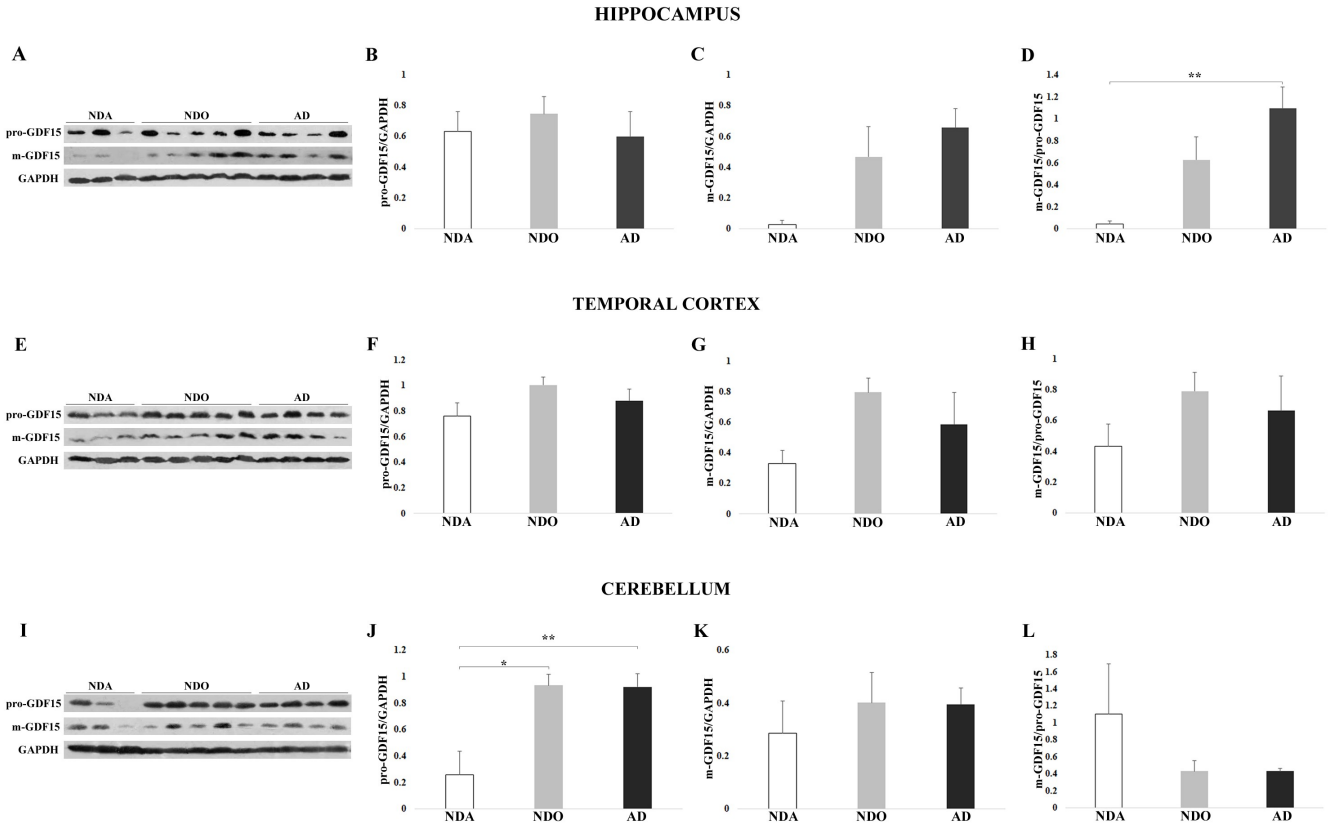
Figure 11. Western blotting analysis of GDF15 in different brain areas. (A) Representative immunoblotting image of pro-GDF15, m-GDF15, and GAPDH in the frontal cortex (Fr), hippocampus (Hi), temporal cortex (Te), parietal cortex (Pa) and cerebellum (Ce). (B–G) Relative protein expression levels of pro-GDF15, m-GDF15, and m-GDF15/pro-GDF15 ratio from (B–D) 2 non-demented old subjects (NDO) and (E–G) 4 AD patients (AD). The bars represent mean ± SE. Student's *t* and one-way ANOVA tests with Bonferroni correction were applied. Western blotting quantification was performed using ImageJ software and normalized to GAPDH expression. *$p < 0.05$.



We then sought for differences in the levels of pro-GDF15 and m-GDF15 between the different groups of subjects (NDA, NDO and AD) within the same brain areas. In the hippocampus, pro-GDF15 was expressed at a similar level in all groups, while m-GDF15 tended to be expressed more in NDO and AD compared to NDA. M-GDF15/pro-GDF15 ratio was significantly higher in AD compared to NDA, similarly to what observed in the frontal cortex (Fig. 12A-D). A similar pattern was observed in the temporal cortex, where m-GDF15 and m-GDF15/pro-GDF15 ratio tended to be higher in NDO and AD compared to NDA (Fig. 12E-H). In the cerebellum, pro-GDF15 expression was significantly higher in NDO and AD compared to NDA, while no significant differences were found for m-GDF15 and m-GDF15/pro-GDF15 ratio (Fig. 12I-L). As regard parietal cortex, only

samples from NDO and AD were available and no significant differences in the expression levels of pro-GDF15 and m-GDF15 were found (data not show).

Figure 12. Western blotting analysis of GDF15 in different brain areas from 3 non-demented adults (NDA), 5 non-demented olds (NDO), and 4 AD patients (AD). Representative immunoblotting image of pro-GDF15, m-GDF15, and m-GDF15/pro-GDF15 ratio and relative protein expression in (A-D) hippocampus, (E–H) temporal cortex, and (I–L) cerebellum. The bars represent mean ± SE. Student's t and one-way ANOVA tests with Bonferroni correction were applied for hippocampus and temporal cortex analyses. Kruskal–Wallis test and Bonferroni correction were applied for cerebellum analysis. Western blotting quantification was performed using ImageJ software and normalized to GAPDH expression. *$p < 0.05$. **$p < 0.01$.



Overall, these results indicate that GDF15 is predominantly expressed by neurons and that this expression is modulated by the presence of AD and, possibly, by extreme aging. Moreover, it appears that this modulation occurs especially in the frontal cortex and hippocampus but not the parietal and temporal cortex, and cerebellum.

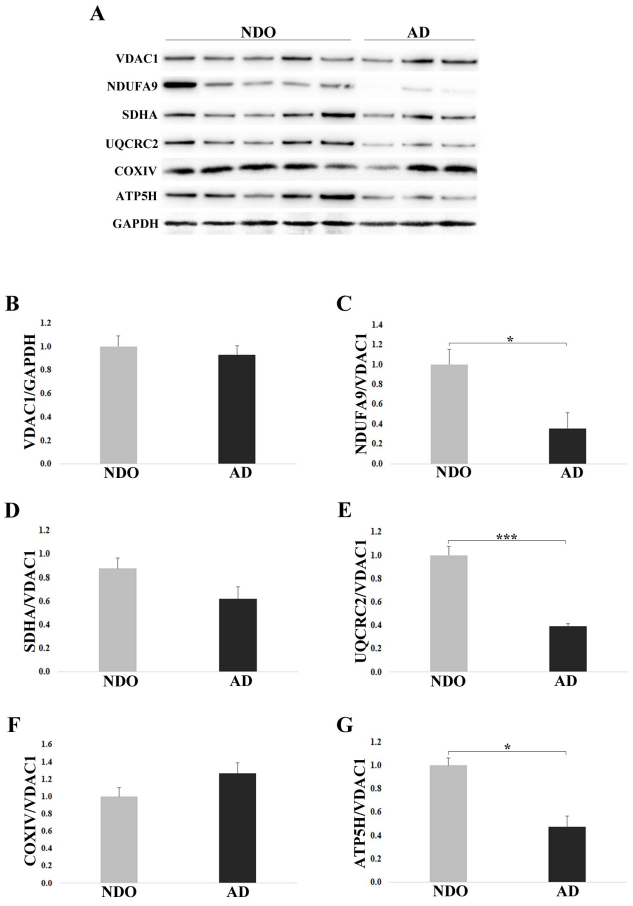
1.4 The expression of proteins of mitochondrial complexes I, III and V is lower in frontal cortex from AD compared to NDO

As AD samples were characterized by higher levels of m-GDF15 and higher *Tp53* expression compared to NDO, we wondered if these samples were also characterized by higher levels of oxidative stress. To check this, we evaluated the level of thiobarbituric acid reactive substances (TBARS), which can be used to assess the level of oxidative stress in biological samples (Aguilar

Diaz De Leon and Borges, 2020), in frontal cortex samples from 4 NDO and 5 AD. No significant differences were found between NDO and AD (NDO: 1.214 ± 0.418 µM/mg of tissue; AD: 1.513 ± 0.506 µM/mg of tissue).

However, as mentioned before, GDF15 is tightly associated to mitochondrial dysfunction, thus we sought to characterize the protein expression of representative subunits of the complexes of the mitochondrial electron transport chain in frontal cortex samples of 11 NDO and 6 AD. First, in order to normalize the expression of the complexes' subunits to the mitochondrial content of the samples, we analyzed the protein level of VDAC1, which can be used as a marker of mitochondrial mass. The level of VDAC1 was similar in NDO and AD samples, suggesting a similar mitochondrial mass in both groups of subjects (Fig 13A, B). NDUFA9 (Complex I; Fig. 13 C), UQCRC2 (Complex III; Fig. 13E) and ATP5H (Complex V; Fig. 13G) expression was reduced in AD compared to NDO. Additionally, SDHA (Complex II; Fig. 13D) and COXIV (Complex IV; Fig. 13F) expression was similar in both groups.

Figure 13. Western blotting analysis of mitochondrial complex subunits in frontal cortex from 11 NDO and 6 AD. (A) Representative immunoblotting image, (B–G) relative protein expression of VDAC1, NDUFA9, SDHA, UQCRC2, COXIV and ATP5H. The bars represent mean ± SE. Student's *t* and one-way ANOVA tests with Bonferroni correction were applied. Western blotting quantification was performed using ImageJ software and normalized to GAPDH expression. Relative expression of OXPHOS proteins was normalized to VDAC1 expression. *$p < 0.05$. ***$p < 0.001$.



As a whole, these data suggest that higher levels of GDF15 are associated to an alteration of the abundance of OXPHOS complexes.

1.5 GDF15 expression is higher in dermal fibroblasts (DFs) obtained from AD compared to DFs obtained from non-demented controls

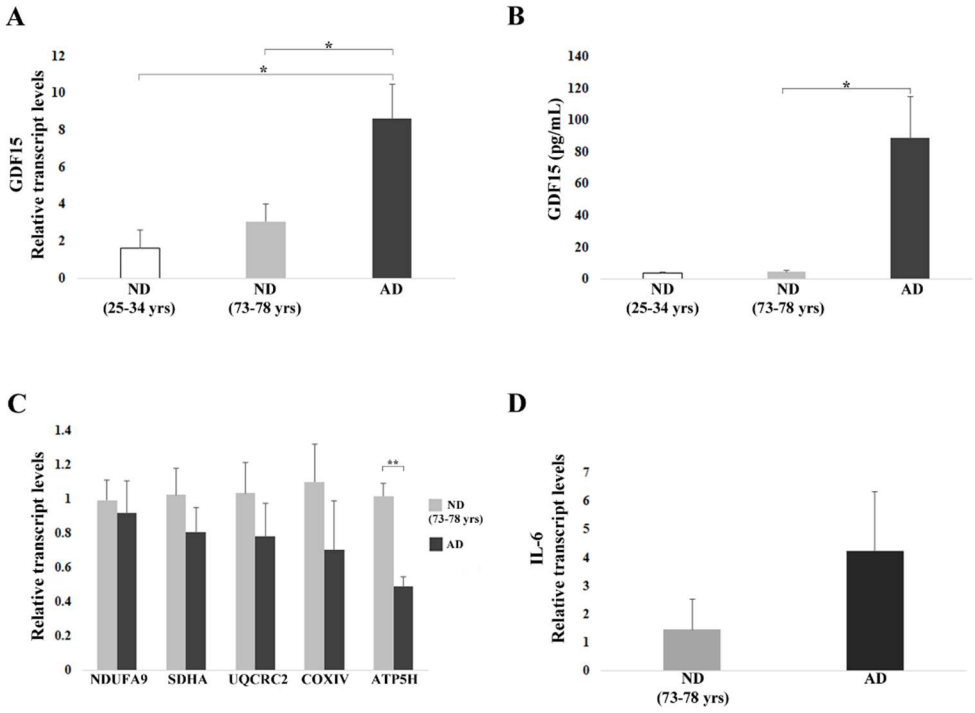
To further characterize the role and the expression of GDF15 in aging and AD, we took advantage of dermal fibroblasts (DFs) obtained from 11 non-demented (ND) subjects (3 from young adults in the age range 25-34 years and 8 from old subjects in the age range 73-78 years) and from 3 AD patients (age range 75-79 years). In fact, according to different studies that proposed that AD could be considered as a systemic disease, DFs recapitulate different typical alterations seen in the brain of AD patients. Specifically, DFs are considered a reliable model to study metabolic and mitochondrial alterations that are typical of AD (Uberti et al., 2002; Pérez et al., 2017).

We analyzed the transcript level of GDF15 by real time RT-PCR. *Gdf15* expression resulted significantly higher in DFs from AD patients compared to DFs from both young and old ND subjects (Fig. 14A). To confirm this result, we also evaluated by ELISA the level of GDF15 protein secreted in the culture medium. GDF15 level in the culture medium was extremely higher in AD DFs compared to ND cells. In particular, the level was significantly higher in AD cells compared to ND (73-78) DFs, whereas, due to a higher experimental variability, the statistical significance was borderline when comparing DFs from AD and ND (25-34) (Kruskal-Wallis test p: 0.067) (Fig. 14B).

We then tested the gene expression of representative subunits of the mitochondrial complexes, in order to evaluate if the reduced abundance of mitochondrial complexes' subunits observed in AD brain was also present in DFs. A trend of reduced expression for all subunits was observed in DFs from AD compared to DF from ND (73-78), even if only *ATP5PD* reached the statistical significance (Fig. 14 C). Furthermore, considering that *Il6* level was higher in the brain of AD patients compared to NDO, we also evaluated the level of this cytokine in DFs. As for the brain, *Il6* level in DFs tended to be higher in AD compared to ND (73-78) (Fig. 14D).

Taken together, these data indicate that DFs from AD show an expression pattern similar to that observed in the brain.

Figure 14. Real-time RT-PCR analyses and ELISA in dermal fibroblasts (DFs) from 3 non-demented young subjects (ND, age range 25–34 years), 8 non-demented old subjects (ND, age range 73–78 years), and 3 AD patients (AD). (A) Relative *GDF15* transcript level and (B) quantification by ELISA of GDF15 protein secreted in the culture medium. (C) Mitochondrial complex subunits (*NDUFA9*, *SDHA*, *UQCRC2*, *COX4I1*, *ATP5PD*) and (D) *IL-6* relative transcript levels. The bars represent mean ± SE. Student's *t* and one-way ANOVA tests with Bonferroni correction were applied for transcript-level analyses. Kruskal–Wallis test was applied for ELISA analysis [AD vs. ND (25–34 years) $p = 0.067$]. *$p < 0.05$. **$p < 0.01$.



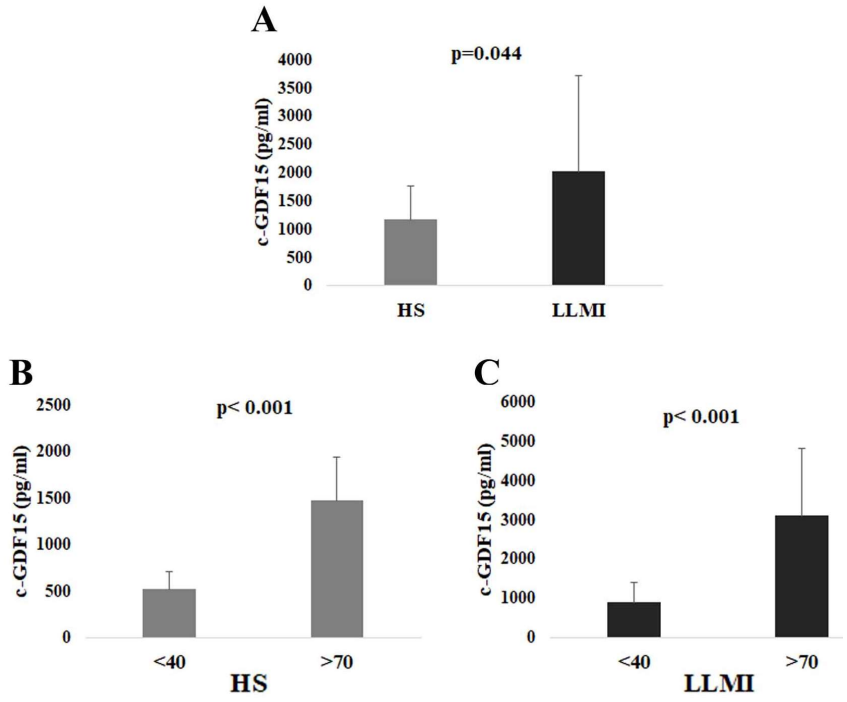
2 Characterization of GDF15 expression in the skeletal muscle of patients with lower limb mobility impairment (LLMI) and healthy subjects (HS)

The second part of the study was focused on analyzing the expression and the role of GDF15 in another typical age-related condition: skeletal muscle atrophy. To do that, we used plasma and skeletal muscle samples from healthy subjects and patients with lower limb mobility impairment, that for their characteristics were comparable to patients with actual sarcopenia.

2.1 Circulating (c-GDF15) and skeletal muscle (SM-GDF15) GDF15 protein levels follow different trends in patients with LLMI (LLMI) and healthy subjects (HS)

In a previous work, we showed that the level of c-GDF15 was elevated in patients with lower limb mobility impairment (LLMI), with respect to healthy subjects (HS) (Conte et al, 2020). In the present study we confirmed these data, *i.e.* LLMI show higher c-GDF15 level compared to HS and, in both groups, >70 years subjects show higher c-GDF15 level compared to <40yrs subjects (Fig. 15A-C).

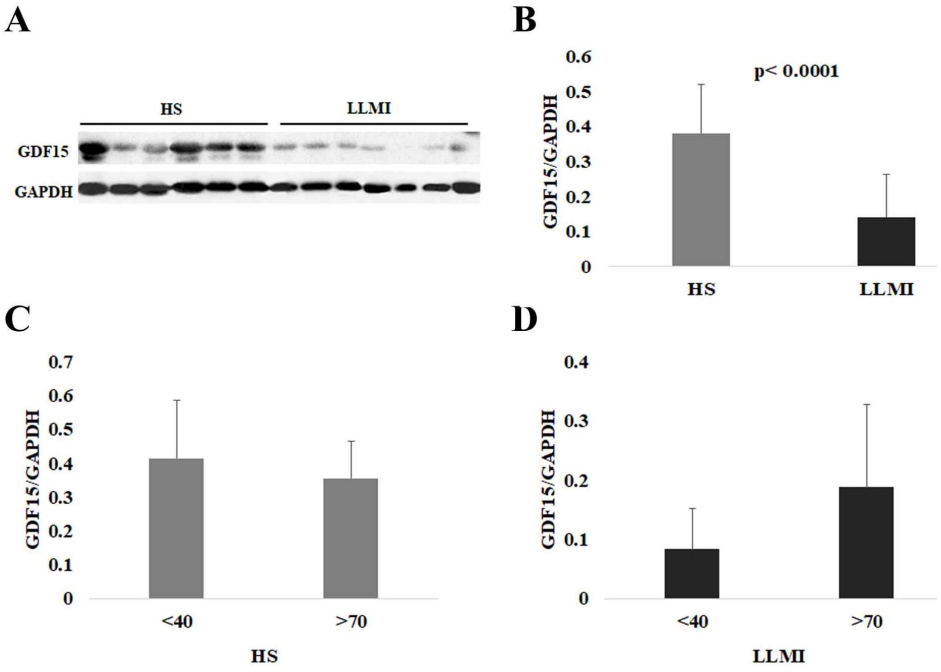
Figure 15. ELISA analysis of c-GDF15 levels in healthy subjects (HS) and patients with lower limb mobility impairment (LLMI). (A) c-GDF15 level in LLMI compared to HS. (B) c-GDF15 level in HS of <40 years of age (<40) compared to HS of >70 years of age (>70). (C) c-GDF15 level in LLMI <40 compared to LLMI >70. Data are expressed as mean ± SD. Mann-Whitney test was applied. Each sample was analyzed in duplicate.



We then wondered if the GDF15 expression trend was the same also in the skeletal muscle. To answer this question, we performed a western blotting analysis to evaluate the protein expression of GDF15 in muscle biopsies of *Vastus lateralis*. The analysis showed that only the precursor, uncleaved form of GDF15 (pro-GDF15) was detectable in the skeletal muscle samples. Quite surprisingly, the level of intramuscular GDF15 (SM-GDF15) was lower in LLMI compared to HS (Fig. 16A, B), following

a different, opposite, trend compared to what was observed for c-GDF15. Moreover, no significant differences were found between <40 and >70 subjects in both HS and LLMI groups (Fig. 16C, D). These results show that GDF15 protein level has different behavior in the plasma and within the skeletal muscle.

Figure 16. Western blotting analysis of GDF15 in skeletal muscle (SM-GDF15). (A) Representative immunoblotting analysis of SM-GDF15 in skeletal muscle biopsies. (B) Relative protein expression of SM-GDF15 in 23 HS and 16 LLMI. (C) Relative protein expression of SM-GSF15 in 10 <40 and 13 >70 HS and (D) in 7 <40 and 9 >70 LLMI. The bars represent mean ± SD. The quantification was performed using Fiji software and normalized to GAPDH expression. Student's t test was applied.



2.2 c-GDF15 correlates positively with markers of inflammation and inversely with markers of muscle atrophy

In our cohort of subjects, we also evaluated multiple biochemical, functional and anthropometric parameters related to inflammation, metabolism and muscle functionality. Thus, we sought for correlations between c-GDF15 and SM-GDF15 and these parameters. In particular the analyzed parameters were: age, BMI, Isometric Quadriceps Strength (IQS), Adiponectin, Leptin, Resistin, Insulin, IL6, IL15, c-PLIN2, IGF-1. The mean values of these parameters are shown in Table 3.

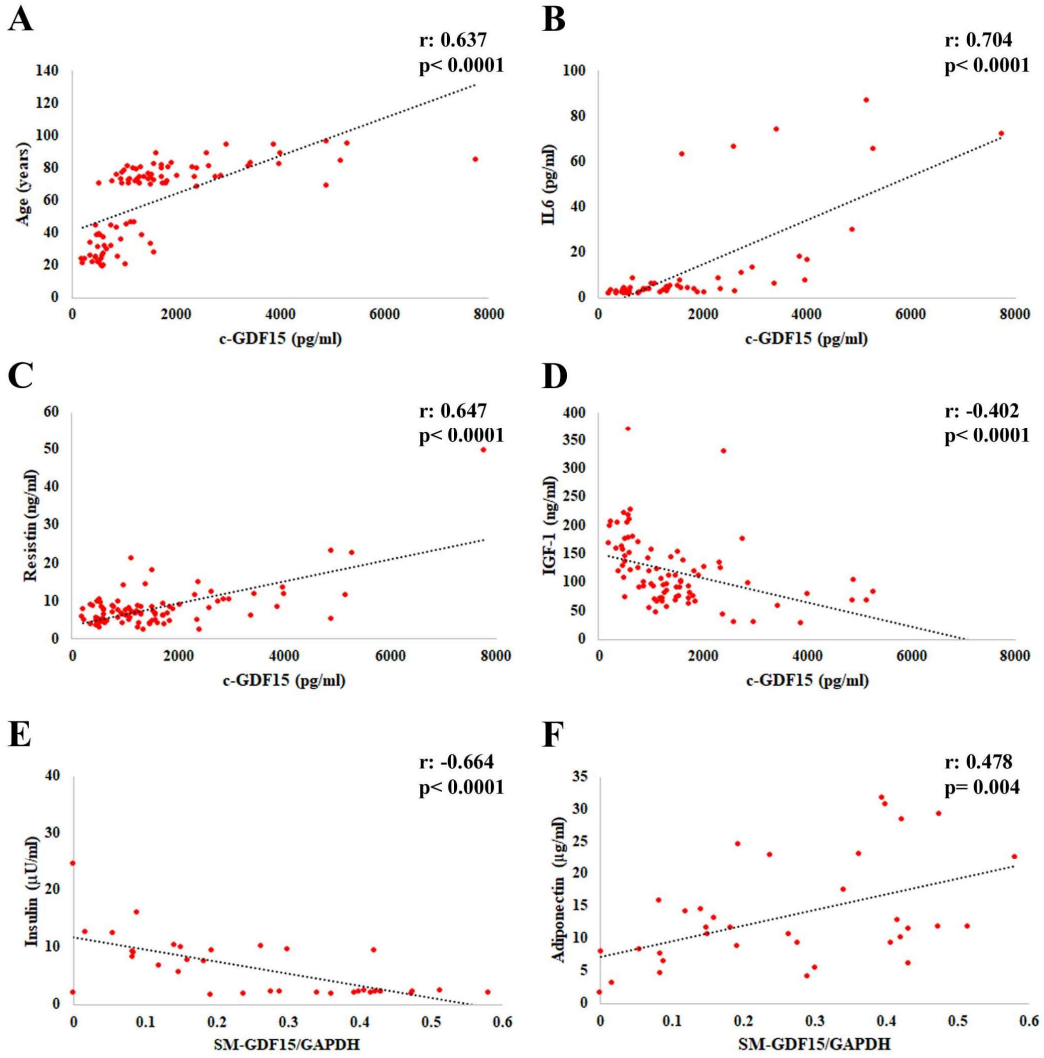
Table 3. Mean values of the parameters measured in HS and LLMI. Data are expressed ad mean ± SE.

	HS (47 subjects)	LLMI (46 subjects)
Age (years)	57.1 ± 3.6	61.3 ± 3.6
BMI (kg/m^2)	24.9 ± 0.6	26.0 ± 0.6
c-GDF15 (pg/ml)	1168.1 ± 87.6	2021.6 ± 249.1
IQS (kg)	146.5 ± 10.4	26.3 ± 1.7
Adiponectin (µg/ml)	13.7 ± 1.2	10.7 ± 1
Leptin (pg/ml)	11.7 ± 1.9	12.2 ± 1.4
Resistin (ng/ml)	7.1 ± 0.4	9.3 ± 1.1

IGF-1 (ng/ml)	122.5 ± 9.7	117.5 ± 8.4
Insulin (µU/ml)	2.1 ± 0.03	12.6 ± 2.1
IL6 (pg/ml)	3.2 ± 0.2	16.7 ± 3.7
IL15 (pg/ml)	2.0 ± 0.1	4.0 ± 1.8
c-PLIN2 (ng/ml)	32.2 ± 4.9	44.7 ± 5.3

When considering all the subjects together (HS+LLMI), we found a positive correlation of c-GDF15 with age, IL6 and Resistin and an inverse correlation with IGF-1. As regard SM-GDF15, we observed an inverse correlation with Insulin and a positive correlation with Adiponectin (Fig. 17A-F).

Figure 17. Linear regression analysis of c-GDF15 with (A) age, (B) IL6, (C) Resistin, (D) IGF-1, and of SM-GDF15 with (E) Insulin, (F) Adiponectin. Pearson correlation coefficient (r) and p-value are shown.



We then considered the two groups of subjects separately. When considering HS group only, a positive correlation of c-GDF15 with age, BMI and IL6 and a negative correlation with IQS, IQS/BMI and IGF-1 were found. For this group, we also had data regarding the body mass composition, and we found a positive correlation of SM-GDF15 with fat percentage and a negative one with lean percentage (27.6% ± 1.3 and 70.4% ± 1.3 of HS body composition on average, respectively) (Table 4).

Table 4. Pearson correlation of c-GDF15 and SM-GDF15 with the biochemical, functional and anthropometric parameters measured in HS. (r: Pearson correlation coefficient; p: p-value).

	c-GDF15		SM-GDF15	
	r	p	r	p
Age	**0.756**	**< 0.0001**	-0.083	0.744
BMI	**0.300**	**0.041**	-0.196	0.436
IQS (kg)	**-0.325**	**0.026**	-0.426	0.078
IQS/BMI	**-0.381**	**0.008**	-0.342	0.165
Fat %	0.165	0.272	**0.517**	**0.028**
Lean %	-0.118	0.435	**-0.527**	**0.025**
Adiponectin	0.122	0.413	0.322	0.192
Leptin	0.154	0.301	0.389	0.110
Resistin	0.131	0.379	-0.005	0.984
IGF-1	**-0.594**	**< 0.0001**	0.059	0.818
Insulin	-0.093	0.534	0.334	0.176
c-PLIN2	0.152	0.330	0.299	0.243
IL6	**0.640**	**< 0.0001**	0.123	0.639
IL15	-0.055	0.104	0.104	0.896
SM-GDF15	-0.199	0.444	-	-

When considering the LLMI group only, we found a positive correlation of c-GDF15 with age, Resistin and IL6 and a negative one with IQS, IQS/BMI, and IGF-1. Moreover, for this group the data about *V. lateralis* thickness were available (1.3mm ± 0.1 on average) and we also found a negative correlation of c-GDF15 with this parameter. As regard SM-GDF15, a positive correlation with IL6 was found (Table 5).

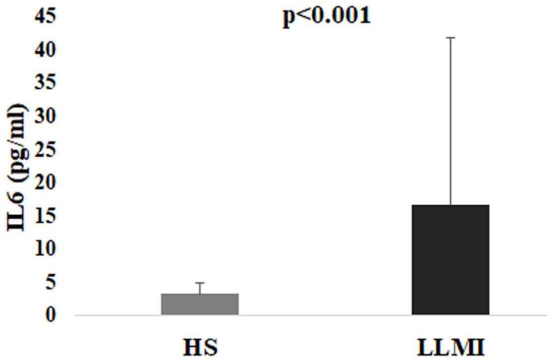
Table 5. Pearson correlation of c-GDF15 and SM-GDF15 with the biochemical, functional and anthropometric parameters measured in LLMI. (r: Pearson correlation coefficient; p: p-value).

	c-GDF15		SM-GDF15	
	r	p	r	p
Age	**0.714**	**< 0.0001**	0.378	0.135
BMI	-0.263	0.081	0.106	0.685
IQS (kg)	**-0.405**	**0.019**	-0.188	0.628
IQS/BMI	**-0.469**	**0.006**	-0.164	0.673
Muscle thickness	**-0.551**	**< 0.0001**	-0.028	0.921
Adiponectin	0.192	0.213	0.284	0.308
Leptin	-0.076	0.623	0.263	0.308
Resistin	**0.689**	**< 0.0001**	0.086	0.743
IGF-1	**-0.403**	**0.013**	-0.205	0.482
Insulin	-0.144	0.350	-0.421	0.092
c-PLIN2	-0.019	0.908	0.291	0.292

IL6	**0.687**	**< 0.0001**	**0.503**	**0.039**
IL15	-0.105	0.493	-0.336	0.188
SM-GDF15	0.366	0.148	-	-

Interestingly, the plasma level of IL6 was significantly higher in LLMI compared to HS (Fig. 18).

Figure 18. Plasma level of IL6 measured by ELISA in HS and LLMI. Data are expressed as mean ± SD. Student's t test was applied. Each sample was analyzed in duplicate.



We also checked for correlations of c-GDF15 and SM-GDF15 with IL15 and c-PLIN2. IL15 is considered a marker of muscle growth (O' Leary et al, 2017) and c-PLIN2 is a marker of adiposity (Conte et al, 2021). No correlations were observed with these parameters. Interestingly, also no correlation was observed between c-GDF15 and SM-GDF15 in both HS and LLMI.

As a whole, these data indicate that c-GDF15 is associated to increased inflammation and decreased muscle strength and could thus represent a biomarker of poor muscle function.

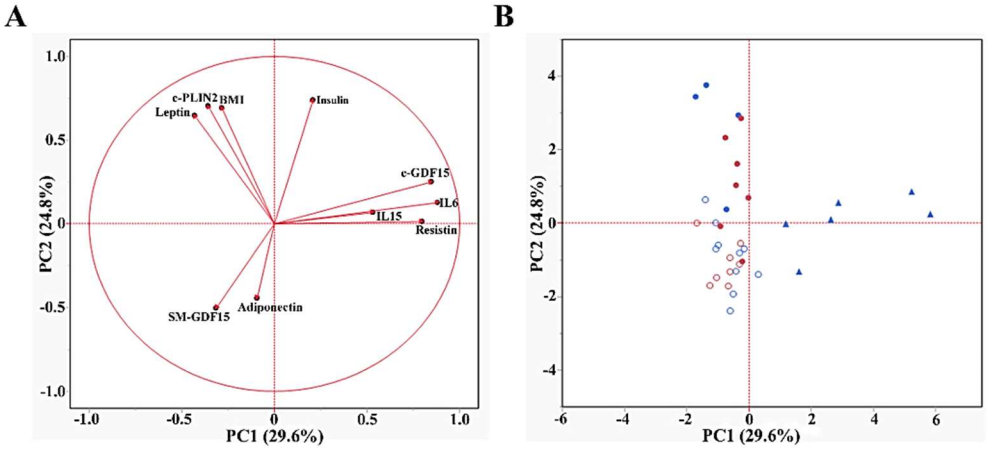
2.3 Principal component analysis (PCA) and canonical discriminant analysis (CDA) well discriminate HS and LLMI

To further clarify the possible diagnostic role of c-GDF15 and SM-GDF15, we performed a principal component analysis (PCA) and a canonical discriminant analysis (CDA).

The PCA was used to explore the effect of the parameters analyzed in HS and LLMI. The first two principal components (PC1 and PC2) were able to describe 54% of the total variation (PC1 29.6%; PC2 24.8%). The loading plot of the PCA showed that all the parameters that we analyzed could be clustered into three main groups (Fig. 19A). The first group included c-GDF15, IL6, IL15 and Resistin, molecules associated to inflammatory responses (Welsh et al, 2003; Park et al, 2017; Perera et al, 2012; Tanaka et al, 2014). The second group included c-PLIN2, Leptin and BMI, all related to lipid metabolism and adiposity (Conte et al, 2021; Obradovic et al, 2021). The third group included Adiponectin, a protein secreted by adipose tissue with anti-inflammatory effects, and SM-GDF15. Interestingly, the score plot of the PCA showed that the main parameters associated to HS were SM-GDF15 and Adiponectin, while LLMI were associated with all the other parameters (Fig. 19B).

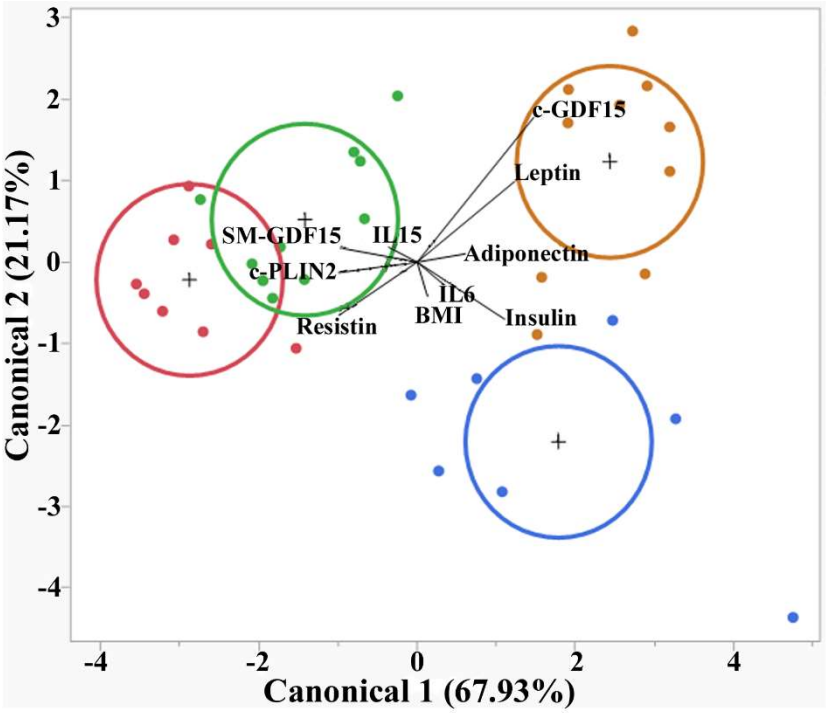
Moreover, LLMI could be further divided by age, with LLMI >85 years being more associated with c-GDF15, Resistin, IL6 and IL15 (Fig. 19B).

Figure 19. Principal component analysis (PCA) in HS and LLMI. (A) Loading plot of the PCA showing the different parameters analyzed. (B) Score plot of the PCA showing the association of the four groups of subjects (<40 HS; >70 HS; <40 LLMI; >70 LLMI) with the different parameters. Empty red circles are HS <40 years; empty blue circles are HS >70 years; filled red circles are LLMI <40 years; filled blue circles are LLMI >70 years; filled triangles are LLMI >85 years.



The CDA showed that the parameters analyzed could well discriminate the subjects into four groups: <40 HS, >70HS, <40 LLMI and >70 LLMI. The canonical 1 was responsible of 67.93% of separation while canonical 2 accounted for 21.17%. In particular, >70 LLMI were separated from all other subjects mainly because of c-GDF15 and Leptin (Fig. 20).

Figure 20. Canonical discriminant analysis (CDA) in HS and LLMI. The groups of subjects are separated based on the parameters analyzed. Red circle: <40 HS; green circle: >70 HS; blue circle: <40 LLMI; yellow circle: >70 LLMI.



We then wanted to determine which of the parameters had the highest discriminatory ability in HS and LLMI. To do that, we performed a receiver operating characteristic (ROC) analysis, calculating the ROC curves. The areas under the curves (AUCs) are reported in table 6 and 7.

Table 6. ROC analysis of metabolic and inflammatory parameters in HS. Areas under the curves (AUCs) are shown. Null hypothesis: true area = 0.5.

Test result variables	Area (AUCs)	Std. Error	p
Adiponectin	**0.734**	0.084	**0.019**
Leptin	0.507	0.103	0.947
Resistin	0.480	0.106	0.843
Insulin	0.023	0.024	0.000
c-GDF15	0.230	0.082	0.007
SM-GDF15	**0.888**	0.062	**0.000**
IL6	0.219	0.089	0.005
c-PLIN2	0.438	0.100	0.529

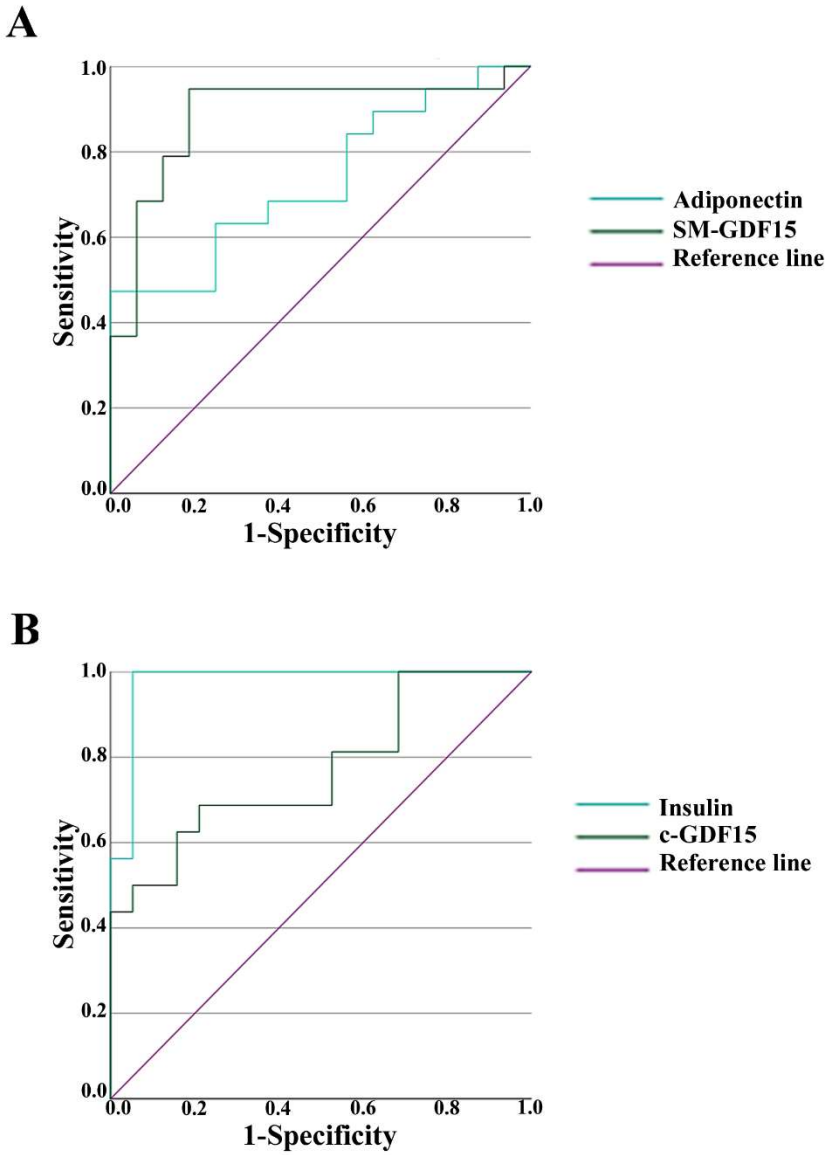
Table 7. ROC analysis of metabolic and inflammatory parameters in LLMI. Areas under the curves (AUCs) are shown. Null hypothesis: true area = 0.5. The test result variable IL6 has at least one tie between the positive actual state group and the negative actual state group, statistic may be biased.

Test result variables	Area (AUCs)	Std. Error	p
Adiponectin	0.266	0.084	0.019
Leptin	0.493	0.103	0.947
Resistin	0.520	0.106	0.843
Insulin	**0.977**	0.024	**0.000**
c-GDF15	**0.770**	0.082	**0.007**
SM-GDF15	0.112	0.062	0.000
IL6	0.781	0.089	0.005
c-PLIN2	0.562	0.100	0.529

SM-GDF15 and Adiponectin were the most performative parameters in discriminating HS (Fig. 21A), while c-GDF15 and Insulin were those for LLMI (Fig. 21B).

Overall, these data indicate that the parameters analyzed were able to discriminate LLMI from HS. In particular, interestingly, c-GDF15 and SM-GDF15 were among the most discriminative parameters for LLMI and HS, respectively.

Figure 21. Receiver operating characteristic (ROC) analysis. (A) ROC curves of SM-GDF15 and Adiponectin in HS. (B) ROC curves of c-GDF15 and Insulin in LLMI.



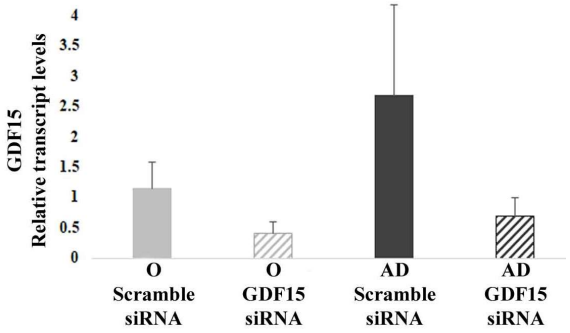
3 *In vitro* knock-down (KD) of GDF15 in human dermal fibroblasts

In order to better clarify the role of GDF15, we took advantage of an *in vitro* model. In particular, we used human dermal fibroblasts (DFs), on which we performed a GDF15 gene knock-down (KD), by using an RNA interference (RNAi) strategy.

3.1 GDF15 KD affects mitochondrial gene expression and morphology, and *Il6* expression

GDF15 gene KD was performed in DFs from both ND (73-78 years) and AD using a combination of siRNA. After GDF15 silencing, we analyzed the expression level of genes coding for subunits of the mitochondrial complexes and *Il6.* First, we checked the silencing efficacy, that resulted robust in both ND and AD DFs (Fig. 22).

Figure 22. Real time RT-PCR analysis of *GDF15* in DFs from 5 old non-demented subjects (O) and 3 AD patients (AD) treated with GDF15 siRNA or scramble siRNA. The bars represent mean ± SE. Student's *t* and one-way ANOVA tests with Bonferroni correction were applied.



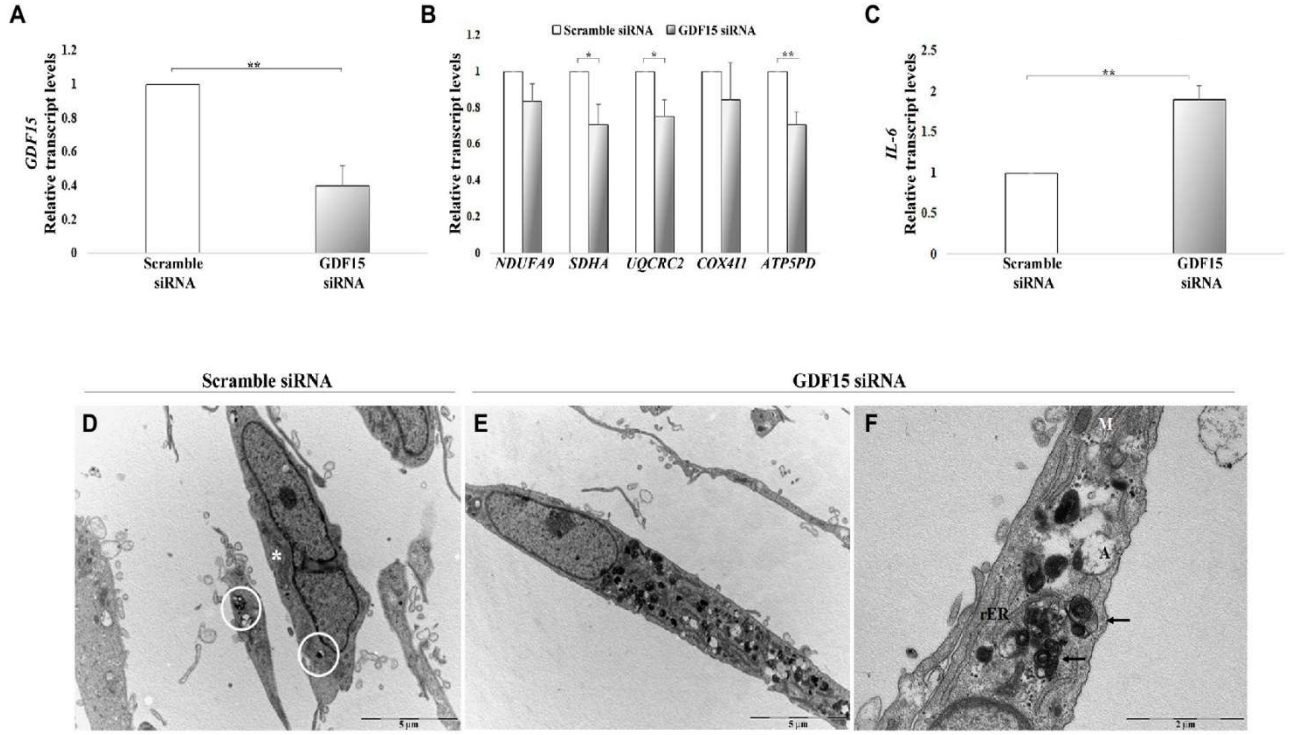
Considering that DFs from ND and AD subjects KD for GDF15 showed similar results, we decided to pool the data together (Fig. 23A).

After GDF15 KD, we observed a significant decrease of the gene expression of mitochondrial complex II (*SdhA)*, III (*Uqcrc2)* and complex V (*Atp5D)* subunits, compared to scramble siRNA (Fig. 23B). Moreover, an increase of *Il6* transcript level was also observed (Fig. 23C). Since we found an alteration of OXPHOS gene expression after GDF15 silencing, we performed a transmission electron microscopy (TEM) analysis to evaluate the mitochondrial ultrastructure after knocking-down GDF15. Interestingly, we observed that GDF15 KD determined the presence of a higher number of degenerated mitochondria. These mitochondria appeared as electron-dense round bodies, presented whorled cristae and were often associated to autophagosomes (Fig. 23D-F).

Taken together, these results support the hypothesis that GDF15 is important in mediating anti-inflammatory responses and has mitochondria-protective effects.

Figure 23. *Gdf15* knock-down (KD) in human dermal fibroblasts (DFs). (A-C) Real time RT-PCR analysis of (A) *Gdf15*, (B) mitochondrial complex subunits (*NDUFA9*, *SDHA*, *UQCRC2*, *COX4I1*, *ATP5PD*), and (C) *Il6* in DFs from 5 ND old in the age range 73-78 years and 3 AD patients pooled together, after *Gdf15* KD obtained with the use of siRNA. The bars represent mean ± SE. Student's *t* and one-way ANOVA tests with Bonferroni correction were applied for transcript-level analyses. *$p <$

0.05. **p < 0.01. (D-F) Transmission electron microscopy (TEM) analysis. The ultrastructural morphology of DFs has been compared in scramble siRNA-treated DFs and GDF15 siRNA-treated DFs. (D) Scramble siRNA-treated DFs show a spindled morphology with long bidirectional projections. In the cytoplasm there are several mitochondria (*) some of which are degenerated (white circle). Scale bar = 5 µm. (E) After GDF15 KD (GDF15 siRNA), DFs accumulate degenerated mitochondria that appear as electron-dense round bodies associated with autophagosomes and altered cristae. Scale bar = 5 µm. (F) Magnification of panel E showing degenerated dense mitochondria with whorled cristae (arrows) and autophagosomes. Scale bar = 2 µm. Legend: M: normal mitochondrion; rER: rough endoplasmic reticulum; A: autophagosomes.



3.2 RNA-Seq analysis shows a global reshape of gene expression after GDF15 KD in DFs

In order to evaluate how GDF15 KD affected global gene expression, and if there was a possible age-dependent pattern, we performed a whole genome RNA Seq on DFs from 3 young subjects and 3 old subjects. The analysis performed showed that the expression of a total of 912 genes was modified upon GDF15 KD with a nominal p value <0.05. In particular, 452 genes were upregulated and 460 were downregulated. Only 84 genes were shared between DFs from young and old subjects, 80 of which changing concordantly (Fig. 24A). Thus, the large majority of genes expression changes is not shared between young and old DFs, showing an age-dependent behavior. Moreover, it is to note that only young DFs passed a more stringent statistical analysis BH-adjusted p value <0.05 and |logFC| >2), supporting the idea that in old age the adaptation to GDF15 depletion could be different from that observed in young cells. The same was true also for shared genes (Tables 8 and 9).

Table 8. RNA-Seq list of differentially expressed genes concordant upregulated in DFs from young (Y) and old (O) donors (HGNC: HUGO Gene Nomenclature Committee; logFC: Log2 fold change; Adj.P-Value: adjusted p-value).

HGNC_symbol	logFC_Y	P.Value_Y	Adj.P.Val_Y	logFC_O	P.Value_O	Adj.P.Val_O
P4HA3	-2.1923	0.0000	0.0017	-1.5491	0.0002	0.2655
EXTL3	-1.7178	0.0000	0.0024	-1.1480	0.0002	0.2964
PLOD2	-1.9941	0.0000	0.0029	-1.0766	0.0096	0.5603
PDE4D	-2.4090	0.0000	0.0030	-1.0674	0.0037	0.4773
TSPAN13	-2.1632	0.0000	0.0034	-1.3491	0.0009	0.3263
ADAMTS6	-1.3637	0.0000	0.0035	-1.2907	0.0000	0.1531
COL10A1	-2.8109	0.0000	0.0046	-1.8200	0.0158	0.6086
LCP1	-4.1753	0.0000	0.0050	-2.0088	0.0199	0.6171
SPP1	-2.3309	0.0001	0.0073	-2.5595	0.0020	0.4400
MYEF2	-1.1623	0.0001	0.0075	-1.1191	0.0042	0.4773
PTPN22	-2.4973	0.0001	0.0080	-1.8268	0.0356	0.7021
MPP4	-2.7286	0.0002	0.0099	-1.9284	0.0014	0.3834
MMP3	-3.2760	0.0003	0.0102	-2.1340	0.0305	0.6816
PDK3	-1.4987	0.0003	0.0102	-1.1475	0.0493	0.7718
GALNT6	-1.3523	0.0003	0.0107	-1.1686	0.0008	0.3101
P3H2	-1.5050	0.0004	0.0116	-1.0127	0.0399	0.7177
MMP1	-3.0466	0.0004	0.0126	-2.3632	0.0168	0.6110
SLC26A4	-3.3096	0.0007	0.0167	-3.2213	0.0431	0.7336
HS3ST3A1	-1.3413	0.0011	0.0201	-1.0392	0.0173	0.6110
STXBP5-AS1	-1.4962	0.0012	0.0216	-1.0677	0.0156	0.6086
ARG2	-1.4628	0.0016	0.0245	-1.3881	0.0097	0.5608
GPR39	-1.3176	0.0019	0.0269	-1.4884	0.0303	0.6816
PDCD1LG2	-1.3894	0.0021	0.0282	-1.0394	0.0283	0.6733
ARHGAP11B	-1.5556	0.0035	0.0364	-1.2157	0.0428	0.7334
LPAR3	-2.2407	0.0039	0.0391	-2.0386	0.0184	0.6144
RTL9	-1.3945	0.0048	0.0440	-1.6841	0.0001	0.2030
RAB3IP	-1.1382	0.0055	0.0474	-1.3600	0.0058	0.4904
MMP10	-1.5979	0.0058	0.0487	-1.4601	0.0486	0.7659
BCL2A1	-2.4364	0.0061	0.0500	-2.2062	0.0121	0.5934

***Note*: Only the genes with BH-adjusted *p* value <0.05 in DFs from young donors are listed in this table. Abbreviations: Adj. *p* value, BH-adjusted *p* value; HGNC, gene name according to HUGO Gene Nomenclature Committee; logFC, log2-transformed fold change; O, in old group; Y, in young group.**

Table 9. RNA-Seq list of differentially expressed genes concordant downregulated in DFs from young and old donors (HGNC: HUGO Gene Nomenclature Committee; logFC: Log2 fold change; Adj.P-Value: adjusted p-value).

HGNC_symbol	logFC_Y	P.Value_Y	Adj.P.Val_Y	logFC_O	P.Value_O	Adj.P.Val_O
SFXN1	1.0844	0.0000	0.0033	1.1452	0.0004	0.3101
ISY1-RAB43	7.9681	0.0000	0.0035	6.8368	0.0412	0.7262
NYNRIN	1.1464	0.0000	0.0035	1.0762	0.0005	0.3101

PPARGC1A	1.5904	0.0001	0.0068	1.1916	0.0022	0.4400
KCNS2	2.0922	0.0001	0.0069	1.6809	0.0009	0.3334
FGD4	1.5381	0.0002	0.0102	1.3743	0.0018	0.4207
PODN	1.7383	0.0003	0.0102	1.6329	0.0007	0.3101
KIT	2.3093	0.0003	0.0115	2.1415	0.0004	0.3101
TMEM35A	1.0086	0.0004	0.0118	1.2386	0.0437	0.7361
MAPT	1.8248	0.0004	0.0119	1.5075	0.0479	0.7638
CD34	1.3339	0.0006	0.0151	2.0415	0.0200	0.6171
DCN	1.0076	0.0008	0.0175	1.1757	0.0035	0.4773
ELFN1	1.9332	0.0010	0.0197	1.3212	0.0165	0.6110
TSPAN11	1.4086	0.0013	0.0220	2.5820	0.0074	0.5296
PTGIS	1.0963	0.0015	0.0232	1.0470	0.0001	0.2030
TAFA5	1.6712	0.0016	0.0245	1.5207	0.0420	0.7315
BRINP1	2.1674	0.0018	0.0261	1.6062	0.0026	0.4530
FAM43A	1.0912	0.0020	0.0273	1.0810	0.0131	0.5934
HMGA2-AS1	1.3809	0.0050	0.0449	1.2484	0.0165	0.6110

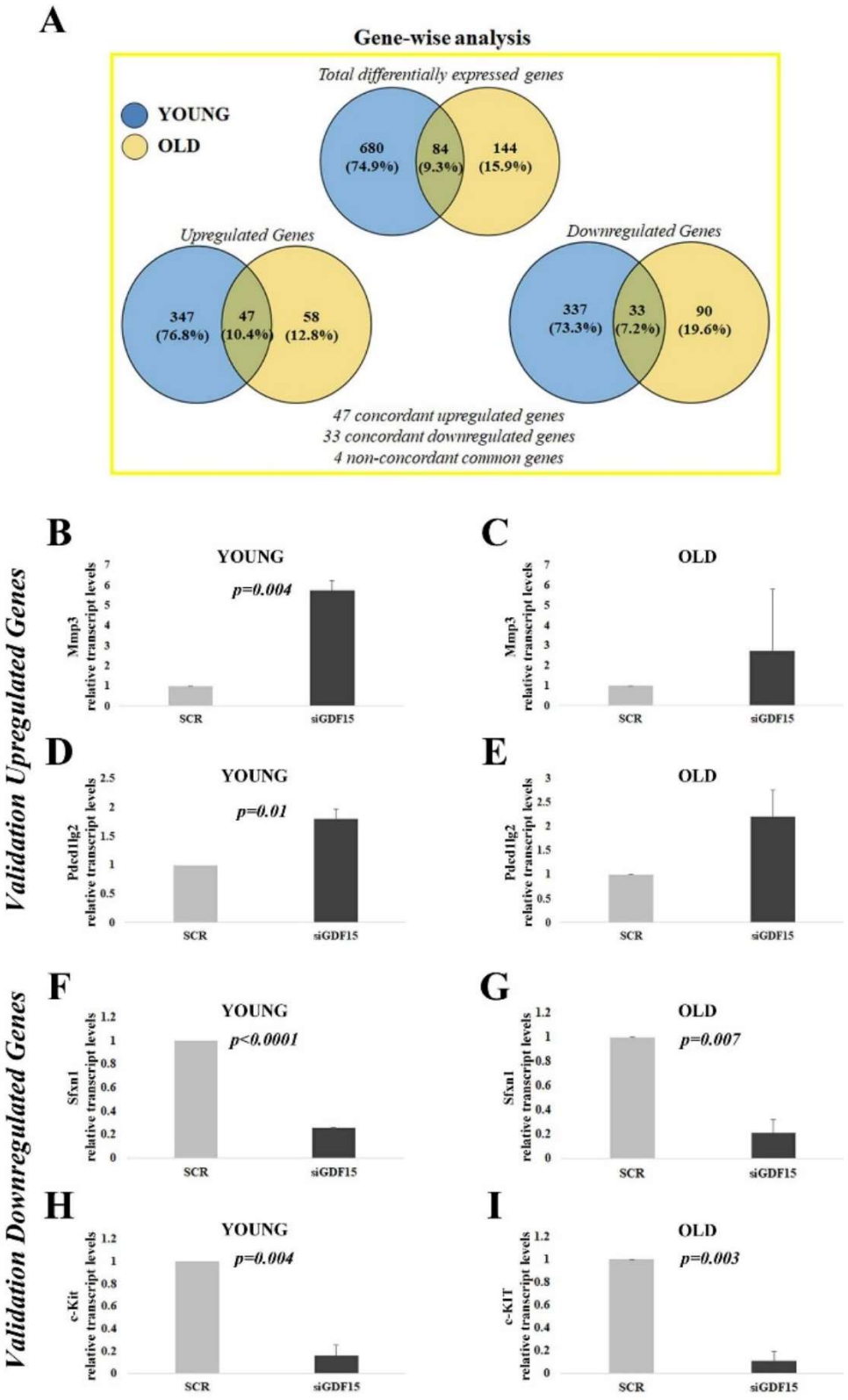
***Note*: Only the genes with BH-adjusted *p* value <0.05 in DFs from young donors are listed in this table. Abbreviations: Adj. *p* value, BH-adjusted *p* value; HGNC, gene name according to HUGO Gene Nomenclature Committee; logFC, log2-transformed fold change; O, in old group; Y, in young group.**

Interestingly, among the upregulated genes upon GDF15 KD, shared between young and old, we found *Mmp1, Mmp3, Mmp10* and *Pdcd1lg2*. Among the shared downregulated genes, we found *c-Kit* and *Sfxn1*. These genes are associated with mitochondrial dysfunction, SASP and cell cycle (Acoba et al, 2021; Foster et al, 2018), suggesting a possible role of GDF15 in regulating these processes.

We then performed a validation analysis, through real time RT-PCR, of selected representative genes. In particular, among the *Mmp* genes, we chose *Mmp3* as it was the one with the highest log2 fold change. We also validated the expression of *Pdcd1lg2, c-Kit* and *Sfxn1*. The real time RT-PCR confirmed the results that emerged from the RNA-Seq analysis (Fig. 24B-I).

Among these genes, at basal level, only the expression of *Sfxn1* was lower in old DFs compared to young (Student's *t* test, p: 0.05).

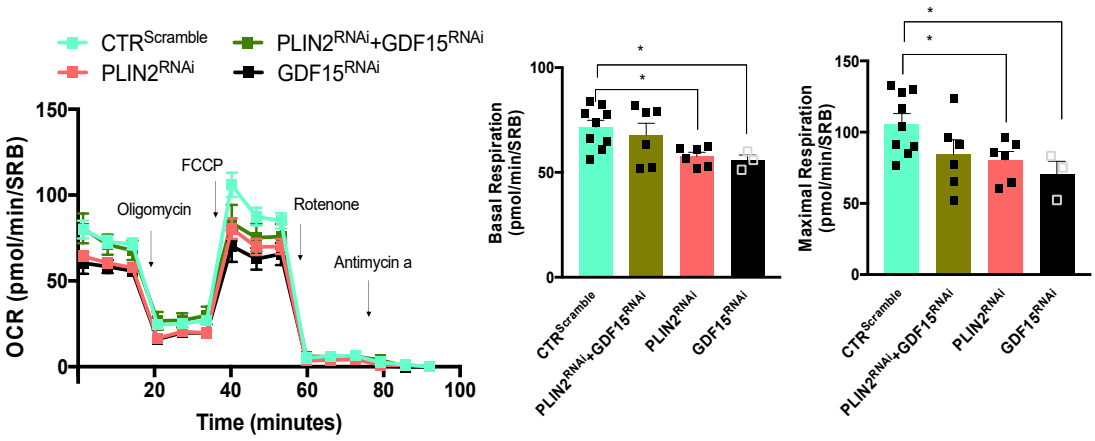
Figure 24. Whole-genome RNA-Seq analysis in DFs. (A) Schematic representation of total and common differentially expressed genes in DFs from 3 young subjects and 3 old subjects, upon GDF15 knock-down. (B-I) Validation, by real time RT-PCR, of selected common upregulated and downregulated genes emerged from the RNA-Seq analysis ((B-C) *Mmp3,* (D-E) *Pdcd1lg2,* (F-G) *c-Kit,* (H-I) *Sfxn1*. Data are expressed as mean ± SE. Student's *t* test was applied.



3.3 PLIN2 (lipid droplet-associated protein) KD in DFs causes GDF15 overexpression and induction of cell senescence *via* GDF15

In our laboratory, we also had another research line focused on analyzing the role of Perilipins (PLINs), and in particular PLIN2, in dermal fibroblasts. PLIN2 is a lipid droplet-associated protein and, in particular, it is involved in lipid droplet biogenesis and lipolysis regulation and is thought to be a key player in lipid metabolism in many cell types and tissues (Conte et al, 2016). We found that PLIN2 KD determined mitochondrial stress and mitochondrial respiration impairment measured by the Sea Horse XFe Mito Stress Test (Chiariello, Rossetti et al, 2024) (Fig. 25). We also evaluated the mitochondrial respiration upon GDF15 KD and double KD of GDF15+PLIN2. As expected considering the alteration of OXPHOS subunits expression (see paragraph 3.1 of Results section), the KD of GDF15 in DFs caused a reduction in both basal and maximal mitochondrial respiration (Fig. 25). Surprisingly, the GDF15+PLIN2 double KD partially abolished the impairment of mitochondrial respiration observed with the two single KD of GDF15 and PLIN2 (Fig. 25).

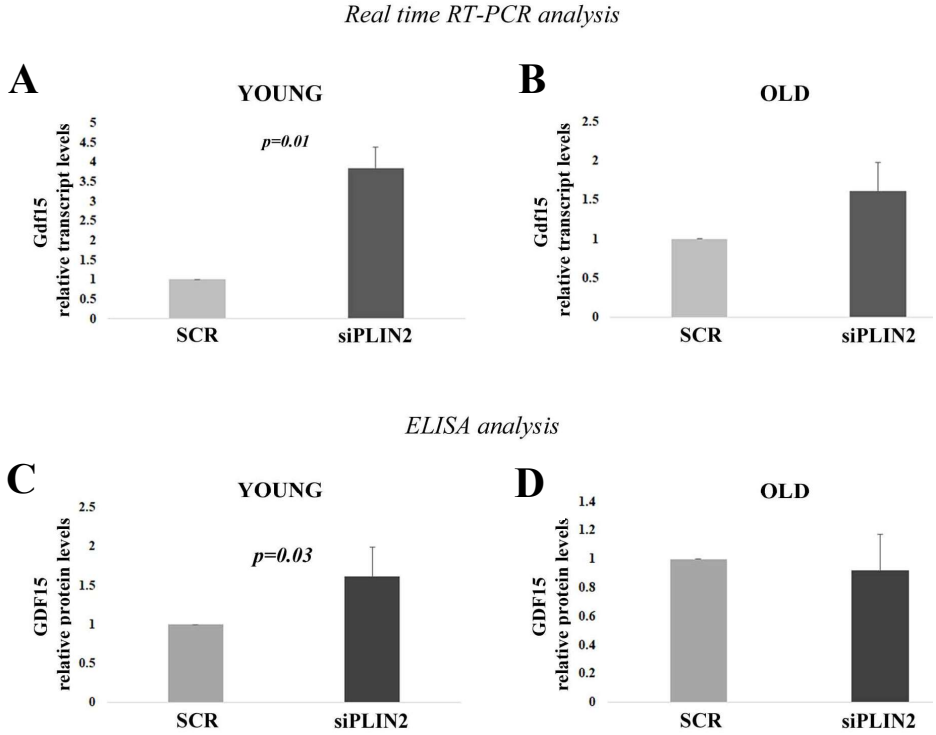
Figure 25. Oxygen consumption rate (OCR) profile of DFs determined upon injection of 1 µM oligomycin, 1.5 µM FCCP, 1 µM rotenone, and 1 µM antimycin A in Seahorse XFe medium. FCCP concentration was previously determined by titration. Data are normalized on SRB absorbance. The determination of basal and maximal respiration calculated from the data obtained in OCR profile is also shown. KD of GDF15 ($GDF15^{RNAi}$), PLIN2 ($PLIN2^{RNAi}$) and GDF15+PLIN2 ($PLIN2^{RNAi}$+ $GDF15^{RNAi}$) were performed. Data are expressed as mean ± SE. Student's *t* test was applied.



Considering the link between GDF15, mitochondrial dysfunction and metabolism regulation, we wondered if GDF15 expression was affected by PLIN2 KD (that caused mitochondrial respiration impairment) in DFs from young and old subjects. We found that, upon PLIN2 KD, *Gdf15* transcript was upregulated in young DFs (Fig. 26 A). In old DFs a non-significant increase of *Gdf15* gene expression was found after PLIN2 KD (Fig. 26B). We then wanted to check if also the GDF15 protein secreted in the culture medium followed the same trend. We thus performed an ELISA analysis and, to eliminate interindividual variability, the increment over the basal level of GDF15 concentration

was calculated for each cell line. The increment of GDF15 level upon PLIN2 KD was statistically significant in young DFs, while no changes were observed in old DFs (Fig. 26C, D).

Figure 26. Analysis of GDF15 levels upon PLIN2 KD (siPLIN2) in DFs from 6 young and 5 old donors. (A, B) Real time RT-PCR analysis of GDF15 in young and old DFs. (C, D) ELISA analysis of GDF15 secreted in the culture medium of young and old DFs expressed as fold change compared to the scramble-treated samples (SCR). Data are expressed as mean ± SE. Student's *t* test was applied.

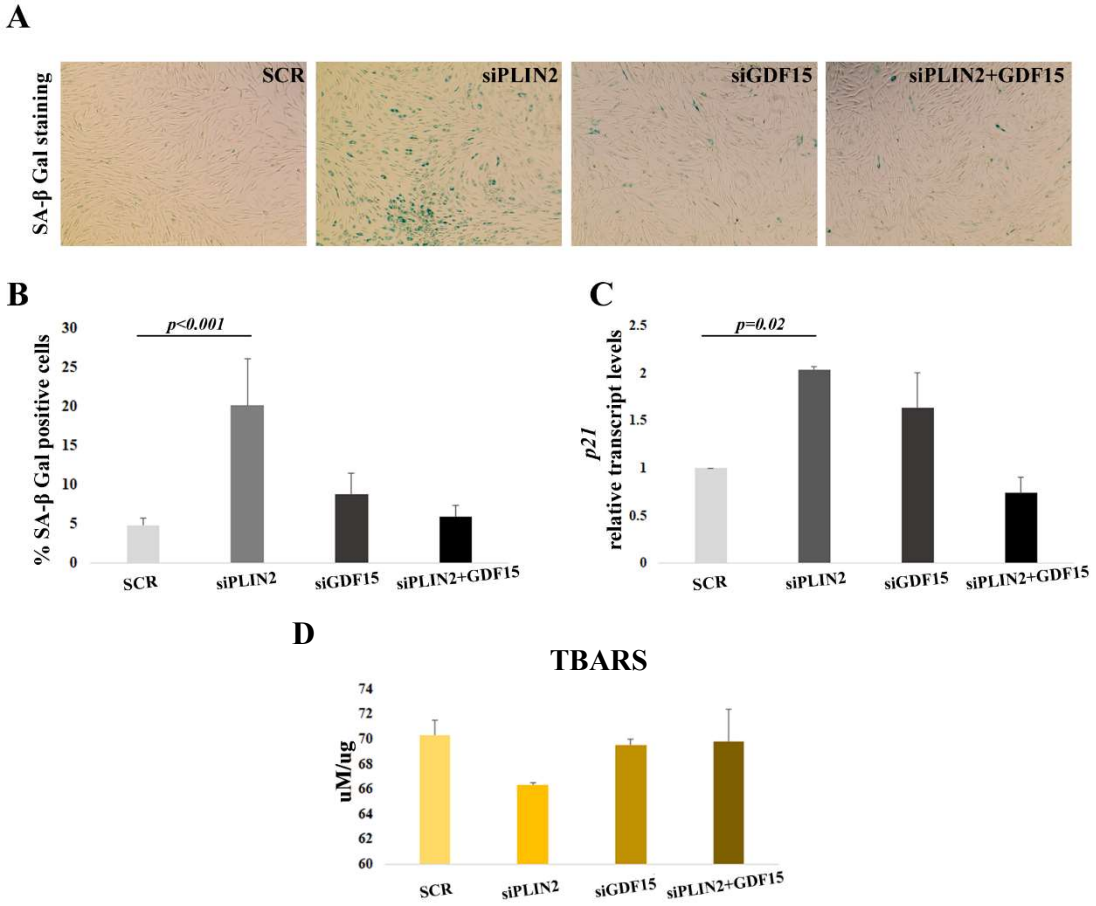


On the contrary, *Plin2* expression was not affected by GDF15 KD (not shown), suggesting that there are no feedback effects of GDF15 on *Plin2* expression.

We then wondered if PLIN2 KD and GDF15 KD were able to induce cell senescence, considering that they caused mitochondrial stress. Thus, we performed a β-galactosidase (β-Gal) colorimetric assay on DFs from young subjects. We found a significant increase in cell senescence induction upon PLIN2 KD (Fig. 27A, B). This result was also confirmed by the increased gene expression of *p21*, considered a marker of cell senescence for its role in cell cycle arrest (Fig. 27C). The KD of GDF15, only caused a slight non-significant increase of cell senescence and *p21* gene expression (Fig. 27A-C). Surprisingly, when we performed a double KD of PLIN2 and GDF15, the induction of cell senescence observed upon PLIN2 KD was partially abolished (Fig. 27 A-C). This result suggests a pivotal role of GDF15 in mediating the induction of cell senescence observed upon PLIN2 KD.

Considering that oxidative stress is able to induce cell senescence, we evaluated the levels of TBARS to have an indication of the oxidative burden after GDF15, PLIN2 and GDF15+PLIN2 KD. No significant differences were found compared to scramble siRNA-treated cells (Fig. 27D).

Figure 27. Cell senescence and oxidative stress analyses in DFs from young subjects. (A) Representative images of β-galactosidase (SA-β Gal) staining in DFs after PLIN2 (siPLIN2), GDF15 (siGDF15), and PLIN2 and GDF15 combined (siPLIN2+GDF15) and scramble (SCR) siRNA treatment. (B) Quantification, performed with Fiji software, of the percentage of cells positive to β-Gal staining after treatment as in A. At least five images per sample were used for quantification and the experiment was performed in three cell lines. (C) Real time RT-PCR analysis of *p21* transcript level in 6 DFs upon GDF15, PLIN2 and PLIN2+GDF15 KD. (D) Thiobarbituric acid reactive substances (TBARS) analysis. Data are expressed as mean ± SE. Student's *t* test was applied.

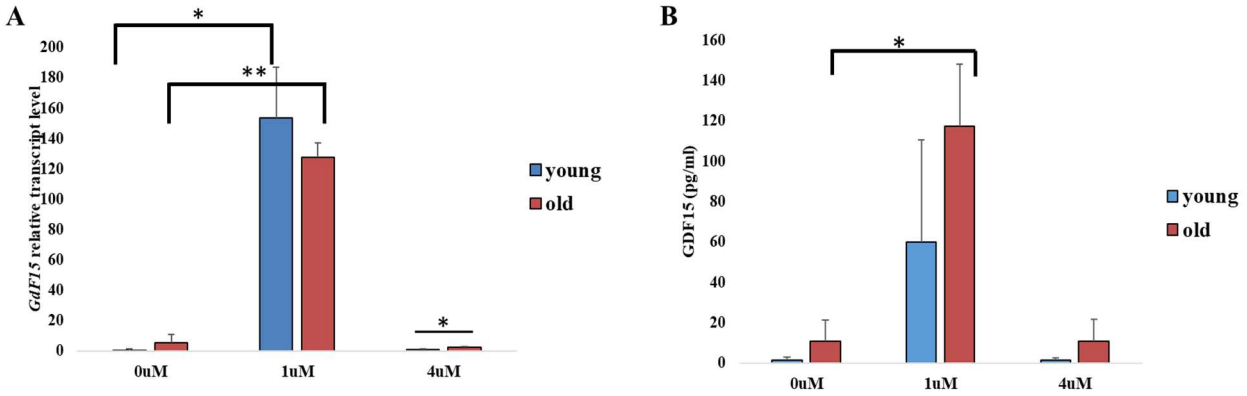


3.4 Doxorubicin treatment in DFs induces GDF15 overexpression

Given the association of GDF15 with mitochondrial stress and, as seen, with the induction of cell senescence, we wanted to check the effect of a well-known senescence-inducing drug, like Doxorubicin (Bielak-Zmijewska et al, 2014), on GDF15 expression in DFs. After a literature search, we decided to test two different doses (1μM and 4μM) of Doxorubicin for 24h on two lines of DFs from young subjects and two from old ones. First, we evaluated GDF15 transcript level and protein secreted in the culture medium after these treatments. We found that *Gdf15* gene expression was significantly upregulated in both young and old DFs at the 1μM dose compared to controls (Fig. 28A). Moreover, *Gdf15* level was higher in old DFs compared to young at 4μM (Fig. 28A). As regard the protein level measured by ELISA test in the cell supernatant, we observed a significantly higher

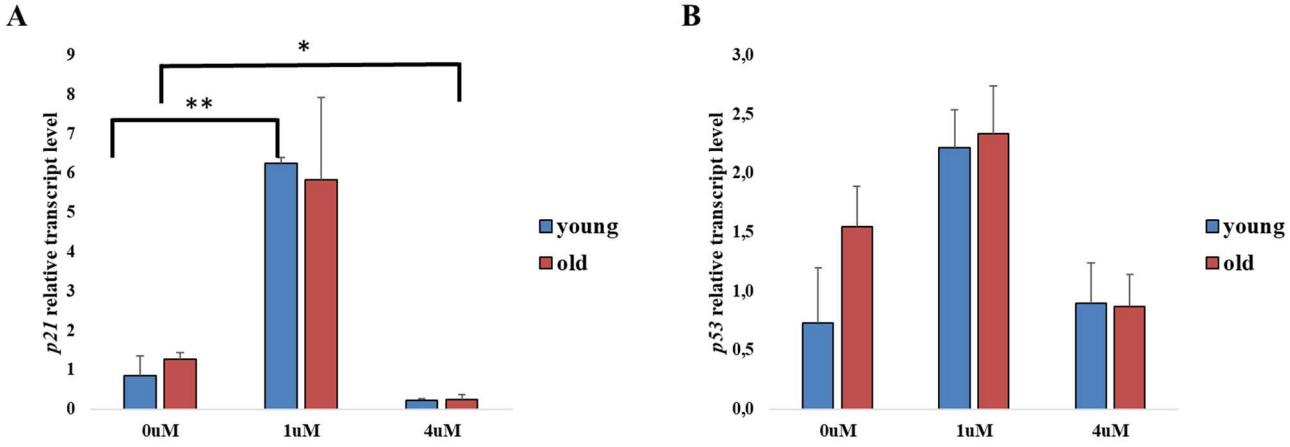
level in old DFs at the dose of 1µM of Doxorubicin compared to untreated cells (Fig. 28B). Overall, the trend was the same observed for the transcript level.

Figure 28. GDF15 level after Doxorubicin treatments in 2 DFs lines from young donors and 2 from old donors. (A) Real time RT-PCR analysis of *Gdf15* after 24h treatment with either 1 or 4µM Doxorubicin. (B) ELISA analysis of GDF15 secreted in the culture medium after the same treatment as in A. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. ***p* < 0.01



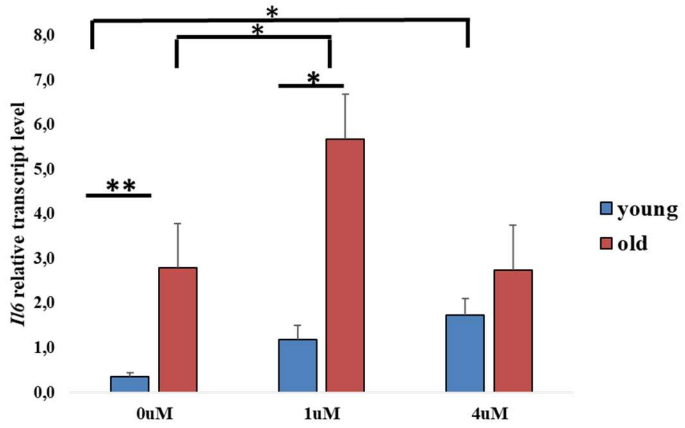
We then checked the expression of two typical senescence and cell cycle-related genes: *p21* and *p53*. The expression of *p21* was higher in young DFs after 1µM treatment with Doxorubicin and, interestingly, lower in old DFs after 4µM treatment compared to control (Fig. 29A). As regard *p53* expression, no significant differences were found (Fig. 29B).

Figure 29. Real time RT-PCR analysis of (A) *p21* and (B) *p53* after 24h treatment with either 1 or 4µM Doxorubicin in 2 DFs lines from young donors and 2 from old donors. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. ***p* < 0.01



Finally, we wondered if the Doxorubicin treatment was able to induce the expression of the cytokine *Il6*. First, we found that *Il6* level was higher in DFs from old compared to DFs from young at 0 and 1µM of Doxorubicin. Moreover, the expression of *Il6* was higher in young DFs after treatment with 4µM of Doxorubicin and in old DFs after treatment with 1µM, compared to untreated cells (Fig. 30).

Figure 30. Real time RT-PCR analysis of *Il6* expression in 2 DFs lines from young donors and 2 from old donors after 24h treatment with either 1 or 4µM of Doxorubicin. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. ***p* < 0.01.



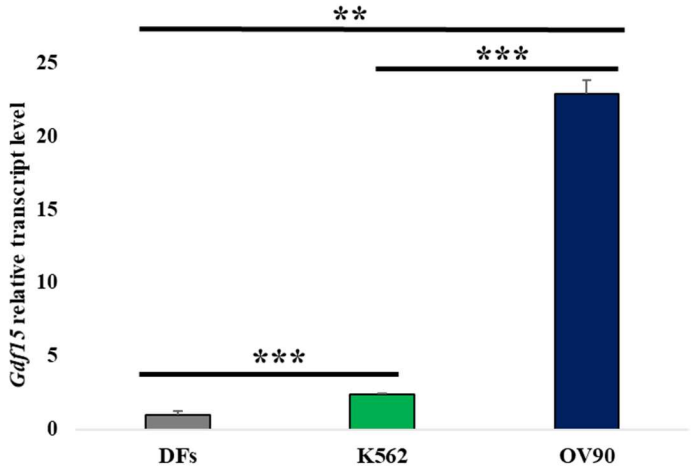
Overall, these data show that Doxorubicin treatment at 1µM is able to induce *Gdf15* overexpression, cell senescence (*p21* increased expression) and increased inflammation in DFs.

4 GDF15 KD and Doxorubicin treatment in tumor cell lines (K562 and OV90)

To explore possible differences in GDF15 effects and roles in cancer cells with respect to normal ones, we took advantage of two additional *in vitro* models: K562 and OV90 cancer cell lines. Specifically, K562 cells are an immortalized myelogenous leukemia cell line and OV90 cells are epithelial-like cells isolated from the ovary of a patient with malignant papillary serous adenocarcinoma. In particular, we wanted to check whether the effects of GDF15 KD and Doxorubicin treatment were similar to what we observed in DFs (see Results paragraph 3).

First, we wondered whether these tumor cell lines had basal *Gdf15* levels similar to normal ones. Interestingly, we found that both K562 and OV90 cell lines were characterized by significantly higher levels of *Gdf15* expression with respect to primary DFs (Fig. 31). This suggests that the biology of cancer cells (at least in this setting) is likely more dependent on GDF15 than normal cells. This hypothesis is at present being investigated.

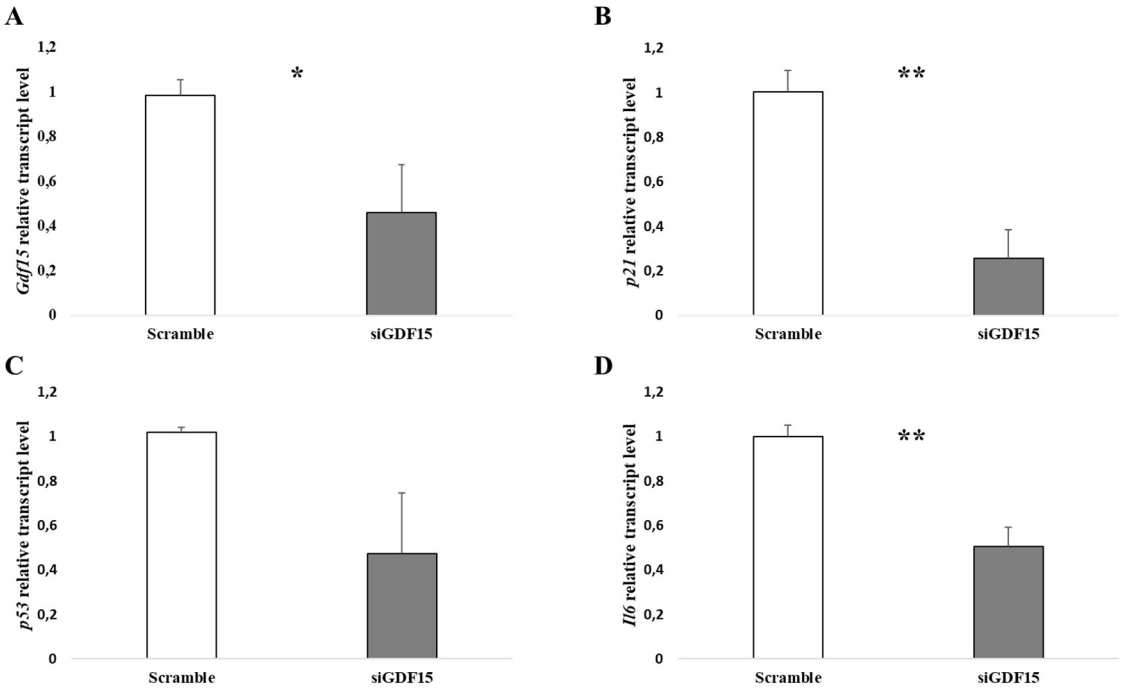
Figure 31. Real time RT-PCR analysis of basal *Gdf15* expression in 2 DFs lines (one young and one old), 3 K562 and 3 OV90 replicates, after 48hrs of culturing. Data are expressed as mean ± SD. Student's *t* test was applied. **$p < 0.01$. ***$p < 0.001$.



4.1 GDF15 KD in K562 and OV90 cell lines

In K562, about 50% of silencing efficiency was reached (Fig. 32A). We sought to analyze the expression of *p21, p53* and *Il6*, to check if GDF15 downregulation in these cells had effects on cell cycle regulation, senescence and inflammation. In contrast to what we observed in DFs, where GDF15 KD determined a (yet non-significant) increase of *p21* expression (see Fig. 27C), in K562 *p21* expression was lower after GDF15 KD compared to scramble siRNA-treated cells (Fig. 32B). *p53* expression tended to be lower in GDF15 KD cells (Fig. 32C), while the expression of *Il6* was lower compared to scramble siRNA (Fig. 32D). This last finding is in contrast to what was observed in DFs, where GDF15 KD caused an increase of *Il6* expression (see Fig. 23C).

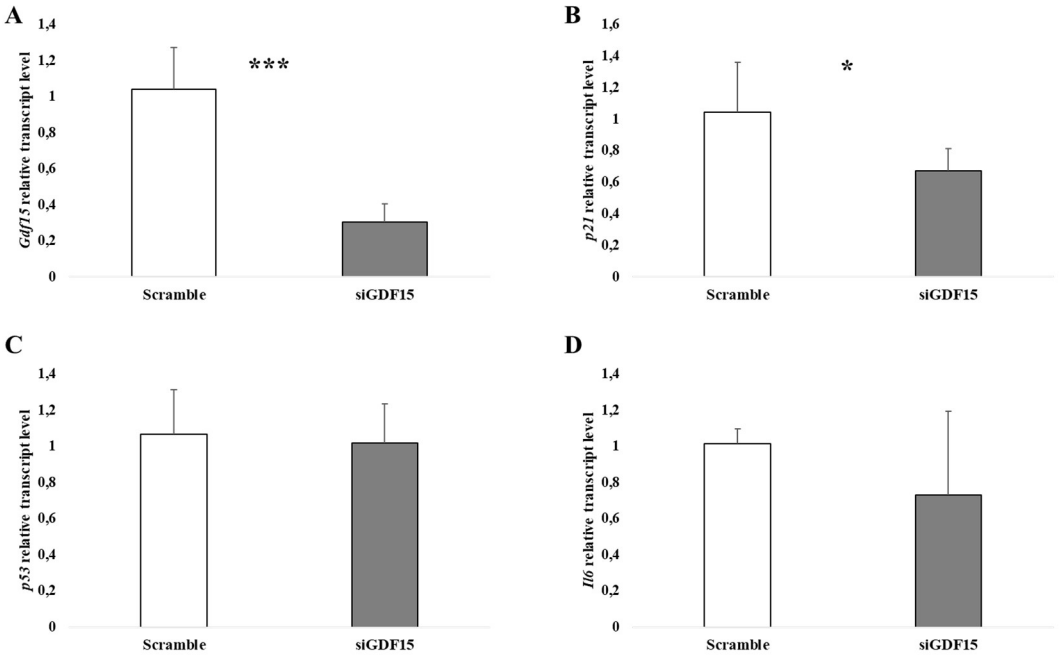
Figure 32. Real time RT-PCR analysis in K562 cells after GDF15 KD (siGDF15). Relative transcript level of (A) *Gdf15,* (B) *p21,* (C) *p53,* (D) *Il6*. Three replicates were performed per each condition. Data are expressed as mean ± SD. Student's *t* test was applied. *$p < 0.05$. **$p < 0.01$.



In OV90 cells, a silencing efficiency of about 70% was obtained (Fig, 33A). As in K562, the KD of GDF15 determined a downregulation of *p21* (Fig. 33B). No statistically significant differences were found for *p53* and *Il6* expression after GDF15 KD, compared to scramble siRNA-treated cells (Fig. 33C, D).

These results are preliminary and further studies are in progress to verify whether GDF15 KD is associated to a decreased survival in both cell lines, and, if so, through what mechanism (mitotic catastrophe? Ferroptosis?). Moreover, we have not yet performed on these cell lines all the analyses that were performed in DFs upon GDF15 KD, such as evaluation of mitochondrial respiration, RNA-Seq analysis and evaluation of cell senescence induction through the β-Gal colorimetric assay. As a whole, at the moment, it looks that in tumor cells GDF15 KD is associated with a decreased inflammatory microenvironment (low *Il6* expression) and diminished control over cell cycle progression (decreased *p21* expression). Overall, these data show that GDF15 downregulation has different effects on human primary cell lines and tumor cell lines, indicating that the role of GDF15 is likely different from normal to tumor cells.

Figure 33. Real time RT-PCR analysis in OV90 cells after GDF15 KD (siGDF15). Relative transcript level of (A) *Gdf15,* (B) *p21,* (C) *p53,* (D) *Il6*. Three replicates were performed per each condition. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. ****p* < 0.001



4.2 Doxorubicin treatment on K562 and OV90 cells

To compare the effect of the treatment with Doxorubicin, a chemotherapeutic drug known to induce mitochondrial damage and, when administered at low doses, cell senescence, with the effects that this treatment had in DFs, we then treated the two tumor cell lines with 1µM or 4µM of Doxorubicin for 24h.

As regard K562 cells, *Gdf15* level increased upon 1µM Doxorubicin treatment, compared to control (Fig. 34A). Interestingly, at 4µM, the expression of *Gdf15* was significantly lower compared to untreated cells (Fig. 34A). We then checked the expression of *p21, p53* and *Il6*. *p21* expression was higher at both 1 and 4µM doses of Doxorubicin compared to control (Fig. 34B). *p53* expression was upregulated at both 1µM and 4µM, but significance was present only for the 1µM dose (Fig. 34C); *Il6* expression level was significantly higher only in 1µM Doxorubicin-treated cells compared to control (Fig. 34D). Overall, Doxorubicin treatment in K562 had similar effects compared to what we observed in DFs (See Fig. 28, 29, 30).

OV90 cells behaved differently compared to K562 and DFs. In fact, *Gdf15* expression only tended to be higher in 1µM treatment (Fig. 35A). The 4µM dose led to a significant decrease of *Gdf15* expression compared to control (Fig. 35A). *p21* expression was higher after 1µM treatment with Doxorubicin and even higher after the 4µM treatment (Fig. 35B). As regard *p53,* its expression was higher in 1µM-treated cells, as observed for K562 (Fig. 35C). Finally, *Il6* expression was higher in both 1µM and 4µM doses of Doxorubicin, compared to untreated cells (Fig. 35D).

The apparently incongruent results obtained with the dose 4µM for the two tumor cell lines are going to be investigated. At the moment, it appears that the 1µM but not the 4µM dose is able to induce cell senescence in K562 cells (and DFs, as seen in the Results paragraph 3.4), while in OV90 cells the 4µM dose appears more effective in inducing cell senescence.

Figure 34. Doxorubicin treatment in K562 cells. Real time RT-PCR analysis of (A) *Gdf15,* (B) *p21,* (C) *p53,* (D) *Il6* after 24h of treatment with 1µM or 4µM of Doxorubicin. Three replicates were performed per each condition. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. **p < 0.01. ****p* < 0.001

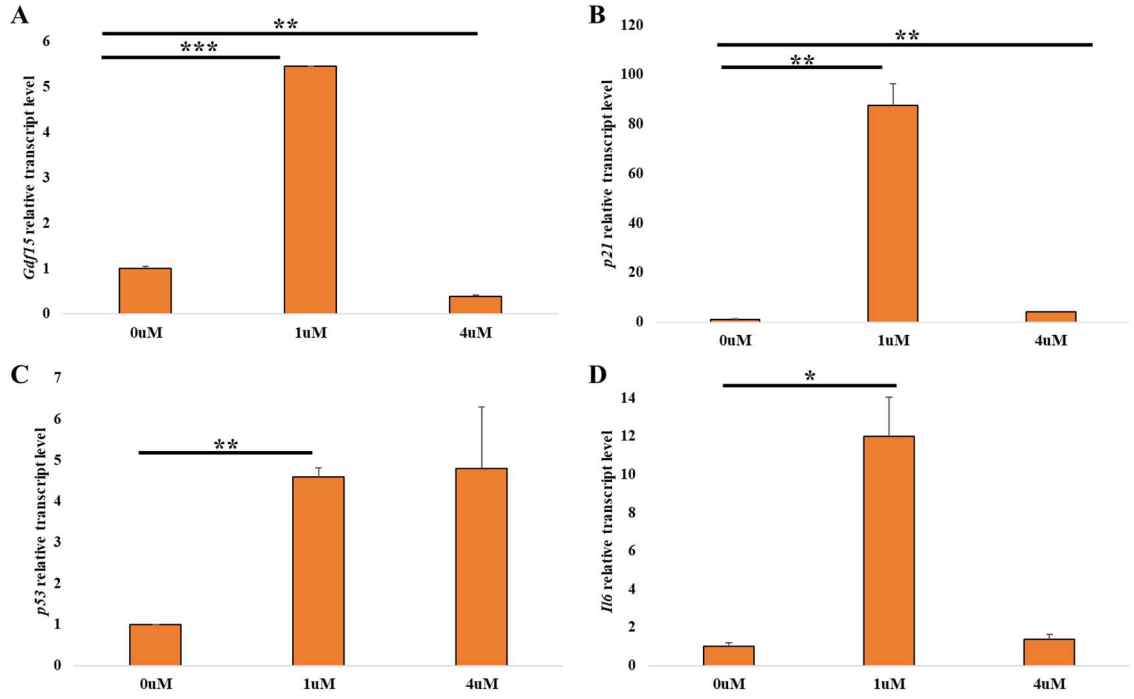
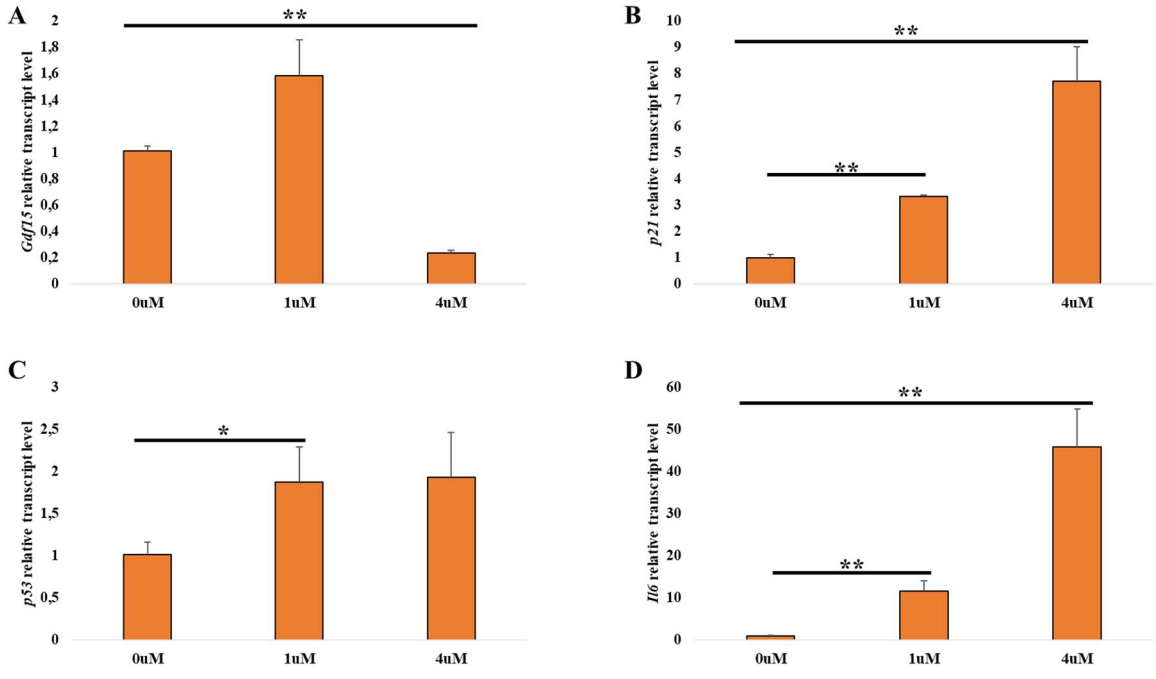


Figure 35. Doxorubicin treatment in OV90 cells. Real time RT-PCR analysis of (A) *Gdf15,* (B) *p21,* (C) *p53,* (D) *Il6* after 24h of treatment with 1µM or 4µM of Doxorubicin. Three replicates were performed per each condition. Data are expressed as mean ± SD. Student's *t* test was applied. **p* < 0.05. **p < 0.01.



Discussion

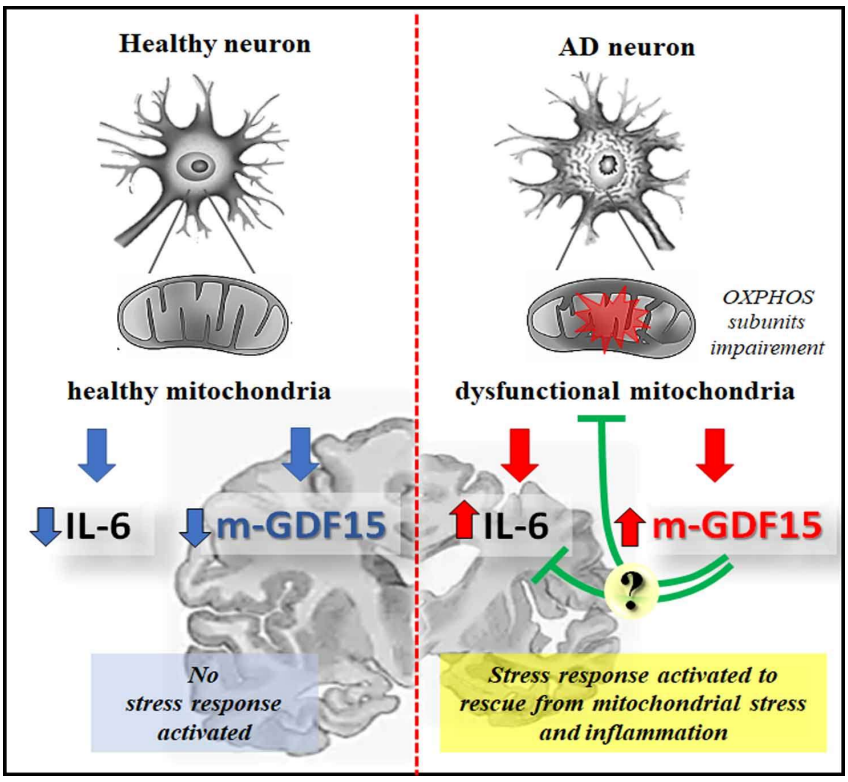
During my PhD course, I have studied the role of GDF15 in different models of aging and age-related diseases, in particular: i) brain tissue and CSF samples from AD patients as compared to age-matched non-demented subjects; ii) plasma and skeletal muscle biopsies from patients with lower limb mobility impairment as compared to subjects with normal mobility; iii) human dermal fibroblasts from healthy subjects of different age and AD patients; iv) two tumor cell lines (K562 and OV90). Part of the results have already been published, while others are still preliminary and further studies are in progress.

As mentioned in the Introduction, Alzheimer's disease (AD) is the most common neurodegenerative disorder, and, in its sporadic form, a typical age-associated disease. We were interested in studying the possible involvement of GDF15 in AD and neurodegeneration. Since GDF15 is mainly known as a secreted protein, we started our investigation from CSF. In a previous work we did not find significant differences in GDF15 plasma levels between AD patients and age-matched controls (Conte et al, 2021). We reasoned that the amount of GDF15 produced in the brain could be too small to have an influence on the plasma level, but it still could be possible to find differences in the CSF. However, in our study we found a similar level of GDF15 in the CSF of AD patients and age-matched non-demented controls. Interestingly, we found a positive correlation between plasma and CSF levels of GDF15, suggesting that, also for CSF, the concentration of GDF15 is most likely determined by the production occurring in organs and tissues other than the brain. No data were available on the expression of GDF15 in brain tissues, thus we performed a thorough analysis on different areas of the brain from subjects of different age as well as AD patients and age-matched non-demented subjects. We have found that GDF15 is expressed and processed into the mature form in different human brain areas, in particular by neuronal cells. Moreover, GDF15 appears to be processed more in the brain of centenarians and AD patients compared to non-demented subjects. This is true in particular for specific brain areas (frontal cortex and – partially - hippocampus). As mentioned before, it is well established in the literature an association of GDF15 with mitochondrial dysfunction and diseases. Accordingly, we observed a reduced expression of representative OXPHOS subunits in frontal cortex of AD patients compared to age-matched non-demented subjects, where the level of m-GDF15 was higher. These results are also well integrated into current theories that foresee a fundamental role for mitochondrial dysfunction in the pathogenesis of AD. In fact, several papers indicate a central role for mitochondrial alterations in the onset and progression of AD (Swerdlow, 2018; Wang et al, 2020). Moreover, mitochondrial dysfunction is also emerging as a trigger for many stress responses aimed at restoring cellular homeostasis, such as UPRmt (Shen et al, 2022). UPRmt

and mitophagy are considered important quality control mechanisms and accordingly, the dysregulation of UPRmt-related proteins leads to neuronal decline during aging (Rugarli and Langer, 2012). Importantly, some of the key transcription factors of GDF15 such as ATF3, DDIT3 (CHOP) and ATF4 are also key components of the UPRmt, possibly making also GDF15 part of this fundamental cellular stress response. Accordingly, an upregulation of the UPRmt, interpreted as a protective response during disease progression has been found in AD (Sorrentino et al, 2017).

We also found a positive correlation of the mRNA level of *Gdf15* in frontal cortex samples with that of *Il6* and *p53*. This association is suggestive of a possible inflammatory response to mitochondrial stress in which GDF15 has a role (Fig. 35) and our *in vitro* data on the KD of GDF15 in human dermal fibroblasts support this hypothesis (see below). Moreover, the fact that we found a higher level of m-GDF15 and a higher m-GDF15/pro-GDF15 ratio in the frontal cortex of centenarians that did not show profound cognitive impairment nor neuropathological features comparable to AD, suggests that likely, GDF15 does not represent the cause of the disease, but rather an attempt to counteract it (Fig. 35). This stress response appears to be well preserved in centenarians, that in fact delay the onset of AD for several years. In such a view, m-GDF15 upregulation in AD brain should be seen as an attempt to cope with the disease, that is in this case unsuccessful.

Figure 35. Schematization of GDF15 expression and roles in healthy and AD neurons. In healthy old subjects, functional mitochondria are associated with a low level of pro-inflammatory cytokines such as IL6, and of m-GDF15 and there is no activation of a stress response. Conversely, in AD neurons, the presence of dysfunctional mitochondria and a reduced expression of OXPHOS subunits is associated to increased level of IL6 and m-GDF15 protein. GDF15 overexpression and increased processing are likely part of a stress response aimed at counteract mitochondrial stress and inflammation (*from Chiariello et al, 2023)*.



Based on our data, it is not clear why these changes in GDF15 expression and processing in AD patients are observed only in frontal cortex and partially hippocampus but not in other brain areas that are also affected by the diseases (temporal cortex and parietal cortex). We hypothesized that this could be due to some kind of "saturation" of GDF15 expression in these areas that did not allow us to detect differences among the groups of subjects. However, the level of GDF15 appeared similar in all brain areas analyzed.

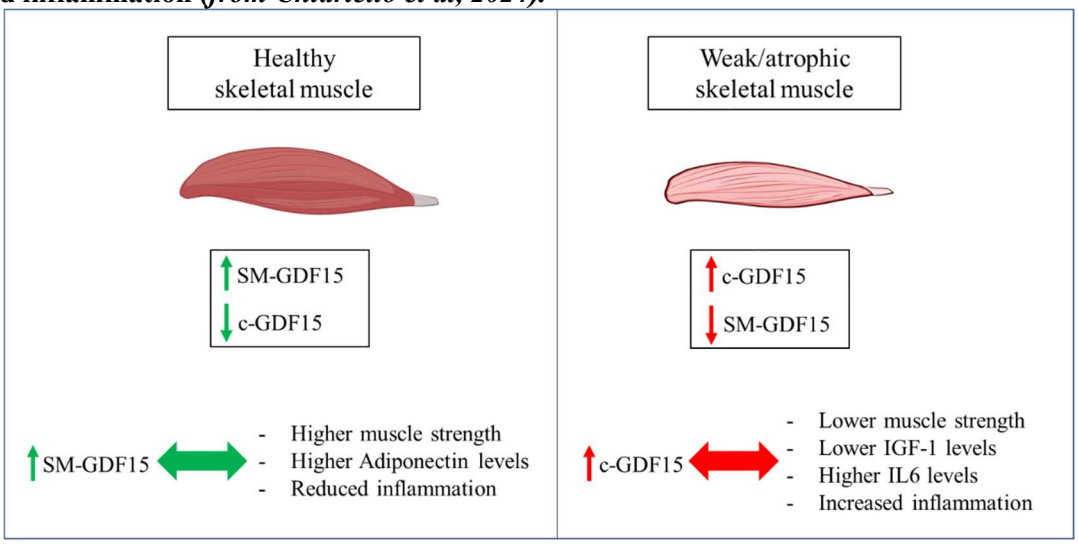
As far as sarcopenia**,** a condition defined by the progressive loss of muscle mass and strength, in particular in old subjects, we wanted to explore the expression and the biological significance of GDF15 in muscle biopsies from healthy subjects (HS) with a normal mobility (i.e. conserved muscle function) or patients with lower limb mobility impairment (LLMI) due to hip dysplasia, leading to muscle atrophy. This has allowed us to compare not only old people (where sarcopenia actually occurs) but also young people, where usually sarcopenia is not present.

In fact, several studies have shown that plasma levels of GDF15 are associated with low muscle function and sarcopenia (Conte et al, 2020; Oba et al, 2020; Yamamoto et al, 2022), but few data on the expression of GDF15 within skeletal muscle are available. First, we were able to confirm that patients with LLMI and muscle atrophy have a higher plasma level of GDF15 (c-GDF15) compared to healthy subjects. Quite surprisingly, we found that the intramuscular protein level of GDF15 (SM-GDF15) was, on the contrary, lower in LLMI patients compared to HS. Moreover, the level of SM-GDF15 did not correlate with the level of c-GDF15 and IQS, suggesting (as also seen in the part of the study regarding brain and AD) that the level of c-GDF15 is likely determined by the production occurring in other organs. Considering that SM-GDF15 level resulted higher in HS, it is conceivable that its production in the skeletal muscle is stimulated by muscle fiber contraction, while inactivity leads to a lower production of SM-GDF15, as shown in Figure 36.

Interestingly, c-GDF15 positively correlates with IL6 and Resistin levels, two markers of inflammation, and inversely with IGF-1 and IQS, suggesting that it is associated to inflammation and decreased muscle function. SM-GDF15 resulted positively correlated with Adiponectin and inversely with Insulin level. In the case of Adiponectin, it is known that it plays a role in maintaining skeletal muscle health and function, in particular by promoting glucose uptake and fatty acids oxidation in the skeletal muscle (Liu and Sweeney, 2014, Krause et al, 2019). Given that a role in promoting lipid oxidation has also been attributed to GDF15 (Zhang et al, 2018), it is possible that the two molecules could have a synergic role in the skeletal muscle. As far as Insulin, it is known that GDF15 improves Insulin sensitivity (Xie et al, 2021, Sjøberg et al, 2023), thus it is possible that an inverse correlation exists between Insulin and GDF15. However, it is to note that existing data regard c-GDF15, but not

SM-GDF15, therefore this hypothesis has to be further validated. Moreover, SM-GDF15 positively correlates with IL6 in LLMI but not in HS. This finding, together with the observation that SM-GDF15 level is lower in LLMI, may suggest that a normal physical activity induces GDF15 expression but not IL6, while chronic inactivity and muscle atrophy may induce IL6 but not (or not so much) GDF15 expression. A limitation of the study regarding this speculation is the fact that intramuscular IL6 expression was not analyzed, however, in absence of overt infections, it can be hypothesized that the larger amount of plasma IL6 derives from contracting muscles. Since GDF15 expression is triggered, among others, by mitochondrial stress, it can be also hypothesized that GDF15 expression is induced in contracting myofibers, where an intense mitochondrial activity is present. On the contrary, inactive muscle may display increased inflammation and a reduced GDF15 expression (Fig. 36).

Figure 36. Schematic representation of the possible diagnostic role of c-GDF15 and SM-GDF15. Higher levels of SM-GDF15 and lower levels of c-GDF15 are present in healthy active subjects, with functional muscles and are associated with reduces inflammation and higher muscle strength and Adiponectin levels. On the contrary, in patients with LLMI and atrophic muscles, a higher level of c-GDF15 and a lower level of SM-GDF15 are observed and are associated with lower IGF-1 levels, higher IL6 levels and increased inflammation (*from Chiariello et al, 2024).*



As regard the missing correlation between c-GDF15 and SM-GDF15, it should be noted that in the skeletal muscle we were only able to detect the immature and non-cleaved precursor form of GDF15 (pro-GDF15), whereas the most found circulating form is the cleaved one. Thus, it is possible that the protein detected in the skeletal muscle, being an immature form, is not proportionally connected with the circulating one.

In further support to the idea that c-GDF15 and SM-GDF15 could have different diagnostic significance in muscle atrophy and sarcopenia, PCA, CDA and ROC analysis indicate that these two parameters have opposite direction and can help discriminating HS from LLMI. In particular, c-GDF15 appears to be the most discriminative parameter for >70yrs LLMI while SM-GDF15 seems

to be associated with muscle activity and functionality, helping in discriminating healthy and active people.

As a whole, the data presented so far indicate that GDF15 is involved in both AD and muscle impairment, however, with a beneficial role. In particular, it seems that the expression of GDF15 in brain has to be seen as an attempt to rescue an underlying mitochondrial stress, while in skeletal muscle, the expression of GDF15 is associated to muscle function. These observations indicate that GDF15, despite being often associated with diseases or even mortality, is not detrimental *per se*, at least not directly, and the high levels of c-GDF15 observed in many pathological conditions have to be considered a consequence of an upstream stress. In order to deepen this aspect, we went further with *in vitro* experiments on different cellular models. The potential beneficial role of GDF15 in maintaining functional mitochondria and counteract inflammation is confirmed by data on **human dermal fibroblasts.** In fact, we found that the KD of GDF15 in these cells led to an increased expression of the cytokine *Il6*, a reduced expression of representative OXPHOS genes, an alteration of mitochondrial ultrastructure with altered *cristae* and the presence of mitochondria in autophagosomes. The induction of mitochondrial stress was also evident with the Sea Horse MitoStress Test, that shows a reduction of basal and maximal respiration upon GDF15 KD. Moreover, the RNA-Seq analysis, shows that GDF15 KD determines the induction of the expression of genes that are part of the SASP. However, the role of GDF15 is more complex and multifaceted, as we also found that PLIN2 KD in DFs induces cell senescence, and this phenomenon appears to be mediated by GDF15. In fact, the double KD of PLIN2 and GDF15 abolished the induction of cell senescence. As a whole, it appears that GDF15 it is not only a component of SASP but is also a potential mediator of cell senescence, at least in specific situations and in presence of specific cellular stresses (such as the KD of PLIN2).

We also had the possibility to explore the effect of GDF15 KD in two different **tumor cell lines: K562** and **OV90**. In these cells the effect of the abrogation of GDF15 appears to be different compared to what we observed in DFs. In fact, upon GDF15 KD we observed a significant reduction of the expression of *p21* in both K562 and OV90 lines and a significant downregulation of *Il6* only in K562 cells. It is thus possible that in over-proliferating tumor cells, GDF15 plays a different role, with a possible specific effect on the regulation of cell cycle. These data are still preliminary and must be further confirmed.

In conclusion, our data suggest that GDF15 has a protective role in situations of cell stress and therefore the statistical associations with diseases must not be misinterpreted as causal relationships. However, the role of GDF15 in cell physiology is likely more complex than expected and we cannot totally rule out that under certain conditions it may play detrimental roles. At a superior layer of complexity (i.e. whole organism), it is likely that the meaning of GDF15 activity is even more intricate, as an activity beneficial for a single cell can turn detrimental for the tissue/organ, making complicate the interpretation of the data collected from clinical studies. Despite these inherent difficulties, GDF15 is emerging as a promising therapeutic target *e.g.* for cancer cachexia (Kim-Muller et al, 2023; da Fonseca et al, 2023) and, based on our and other data, possibly also cell senescence could be counteracted by blocking its expression, at least in certain situations. It is not excluded however that, given its beneficial effects, its direct administration could be envisaged as a therapeutic strategy, too. At present, I'm performing studies aimed at test both hypotheses and hopefully will obtain some interesting results that will help clarify these aspects of GDF15 biology.

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