Review

# Biomarkers of aging in frailty and age-associated disorders: State of the art and future perspective

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## ABSTRACT

According to the Geroscience concept that organismal aging and age-associated diseases share the same basic molecular mechanisms, the identification of biomarkers of age that can efficiently classify people as biologically older (or younger) than their chronological (i.e. calendar) age is becoming of paramount importance. These people will be in fact at higher (or lower) risk for many different age-associated diseases, including cardiovascular diseases, neurodegeneration, cancer, etc. In turn, patients suffering from these diseases are biologically older than healthy age-matched individuals. Many biomarkers that correlate with age have been described so far.

*Abbreviation:* AD, Alzheimer's Disease; AI, Artificial Intelligence; AIS, Acute Ischemic Stroke; ALS, Amyotrophic Lateral Sclerosis; ARD, Age-Related Diseases; BMI, Body Mass Index; CAD, Coronary Artery Disease; CJD, Creutzfeldt-Jakob isease; CKD, Chronic Kidney Disease; CRP, C-Reactive Protein; CoViD-19, Corona Virus Disease-19; CSF, Cerebrospinal Fluid; CVD, Cardiovascular Disease; DAMP, Damage-Associated Molecular Pattern; DL, Deep Learning; DNAm, DNA Methylation; ER, Endoplasmic Reticulum; EWAS, Epigenome Wide Association Studies; EVs, Extracellular Vesicles; FGF21, Fibroblast Growth Factor 21; FTD, Frontotemporal Dementia; GDF15, Growth Differentiation Factor 15; GM, Gut Microbiota; gp130, Glycoprotein 130; HCT, Hematopoietic Cell Transplantation; HD, Huntington's Disease; HF, Heart Failure; HMGB1, High Mobility Group Protein B1; HN, Humanin; HUVECs, Human Umbilical Vascular Endothelial Cells; iAge, Inflammatory Clock of Aging; IL, Interleukin; IFN-γ, Interferon Gamma; MACE, Major Adverse Cardiovascular Events; MAPT, Multidomain Alzheimer Preventive Trial; MCI, Mild Cognitive Impairment; miRNA, microRNAs; ML, Machine Learning; MMSE, Mini Mental State Examination; MS, Multiple Sclerosis; mtDNA, mitochondrial DNA; MWAS, Methylation Wide Association Studies; NETs, Neutrophil Extracellular Traps; Nfs, Neurofilaments; NfL, Neurofilament Light Chain; OP, Osteoporosis; PCR, Polymerase Chain Reaction; PD, Parkinson Disease; PGRN, Progranulin; RA, Rheumatoid Arthritis; ROS, Reactive Oxygen Species; sTNFR1, Soluble Tumor Necrosis Factor Alpha Receptor 1; SASP, Senescence-Associated Secretory Phenotype; SCD, Subjective Cognitive Decline; SCFA, Short Chain Fatty Acids; ST2, Suppression of Tumorigenicity 2; T2D, Type 2 Diabetes; TBI, Traumatic Brain Injuries; TMAO, Trimethylamine N-Oxide; TNFα, Tumour Necrosis Factor alpha; UPRmt, Mitochondrial Unfolded Protein Response; WMH, White Matter Hyperintensity.

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The aim of the present review is to discuss the usefulness of some of these biomarkers (especially soluble, circulating ones) in order to identify frail patients, possibly before the appearance of clinical symptoms, as well as patients at risk for age-associated diseases. An overview of selected biomarkers will be discussed in this regard, in particular we will focus on biomarkers related to metabolic stress response, inflammation, and cell death (in particular in neurodegeneration), all phenomena connected to inflammaging (chronic, low-grade, age-associated inflammation). In the second part of the review, next-generation markers such as extracellular vesicles and their cargos, epigenetic markers and gut microbiota composition, will be discussed. Since recent progresses in omics techniques have allowed an exponential increase in the production of laboratory data also in the field of biomarkers of age, making it difficult to extract biological meaning from the huge mass of available data, Artificial Intelligence (AI) approaches will be discussed as an increasingly important strategy for extracting knowledge from raw data and providing practitioners with actionable information to treat patients.

## 1. Introduction

A growing interest for biomarkers related to the aging process has emerged in the last years (Bürkle et al., 2015; Moreno-Villanueva and Bürkle, 2019), and many have been identified and validated, including epigenetic clocks (Horvath, 2013b; Hannum, Gensous et al., 2013, 2022; Belsky et al., 2022), N-glycome changes (Vanhooren et al., 2007; Vanhooren et al., 2010; Paton et al., 2021; Fan et al., 2023), and miRNA (Olivieri et al., 2017). Beside the indisputable importance of identifying markers able to correlate with either chronological (i.e. calendar age) or biological age, it is likewise important to understand whether and to what extent these biomarkers of age have a diagnostic/prognostic value for patients affected by age-related diseases. This query stems as a consequence of the Geroscience concept (Sierra, 2016). Based on this theoretical model, aging process is the main culprit of age-related diseases development and progression (Fulop et al., 2018). Geroscience shifted the focus away from specific diseases and their role in mortality, toward an assessment of overall health in which the individual's physiology will determine his/her overall risk for chronic diseases, irrespective of which disease is more likely to affect the individual based on genetic and/or environmental factors (Sierra et al., 2021). According to this conceptualization, circulating biomarkers related to the aging process should be significantly associated with several different age-related diseases and conditions (Franceschi et al., 2018). Many biomarkers studied so far fit with this requirement, however, this makes them not very specific. In this framework, in contrast with single blood marker analysis, the multi-biomarker approach, by using traditional or new circulating molecules, might have the potential to enhance the risk stratification in elderly patients affected by chronic diseases and capture subtle nuances such as biological age and pace of aging (Belsky et al., 2015). These composite biomarkers include as an example the so-called inflammatory clock of aging (iAge), a metric for chronic inflammation derived from the level of circulating immune proteins likely reflecting an individual's inflammatory burden (Sayed et al., 2021). However, these composite biomarkers are usually derived from one or more high-throughput techniques that make them quite expensive and not so easy to analyze. So far, these shortfalls have limited their bench-to-bedside translation.

In this review we will discuss recent research developments on age biomarkers with special regard for their involvement in frailty and multimorbidity. We will not list and describe all possible biomarkers of age, rather we will focus on specific ones, emerging as important or promising for a number of conditions strictly linked to frailty, such as inflammaging, mitochondrial dysfunction, neurodegeneration, osteoporosis and sarcopenia. We will finally discuss the new frontier of Artificial Intelligence (AI) as a tool to identify new pathways/mechanisms/markers related to frailty and multimorbidity in old age.

## 2. Markers of inflammaging

Inflammaging, defined as a state of chronic low-grade systemic inflammation, constitutes one of the main biological features of human aging (Franceschi et al., 2018). The upstream driver of this process is likely immunosenescence, the age-related dysregulation of immune response, which determines i. the repeated activation of inflammatory pathways, ii. the accumulation of senescent cells characterized by the so-called senescence-associated (pro-inflammatory) secretory phenotype (SASP) (Coppé et al., 2008); iii. the increased production of "cellular garbage" due to the missed clearance of aged/damaged cells that in turn activate pro-inflammatory innate immune response ("garb-aging", Franceschi et al., 2017). Since the definition of these complex biological phenomena in the early 2000 s (Franceschi et al., 2000), the scientific community has produced a considerable effort in the search for biomarkers potentially related to inflammaging and its regulatory counterpart, anti-inflammaging. Various molecules have been proposed to date, however the results have not always been satisfactory and sometimes are conflicting, making it impossible to reach a broad consensus (Al Saedi et al., 2019). Nonetheless, it is possible to identify interleukin-6 (IL-6), IL-1, C-reactive protein (CRP) and tumour necrosis factor alpha (TNFα) as the most relevant circulating markers related to inflammaging.

The production and release of IL-6 and TNFα from the inflammatory acute-phase cells have been associated with typical geriatric syndromes, such as frailty and its predisposing conditions: sarcopenia, reduced functional capacity and mood disorders (Darvin et al., 2014; Maes et al., 1997; Soysal et al., 2016; Visser et al., 2002). Notably, both cytokines stimulate the liver expression of CRP, another widely shared and easily measurable inflammatory biomarker of unsuccessful aging. In fact, high levels of CRP have also been associated with frailty and its components (Samson et al., 2019). Contrarywise, other research groups have found no significant difference in the concentrations of these pro-inflammatory markers in frail subjects (Alberro et al., 2021). Furthermore, they seem unable to predict transition of older adults from robustness to different degrees of frailty (Baylis et al., 2013), thus concurring to fuel scepticism about the usefulness of IL-6, TNFα and CRP in screening, early identification, and prevention of older adults at increased risk of frailty. Indeed, the major limitation of these biomarkers is their non-specificity within a context of frailty, as their levels are affected by a plethora of stimuli, including inflammatory, neoplastic and infectious diseases. Importantly, they cannot provide information about the specific underlying stimuli which led to their release, thus complicating their interpretation. Moreover, it should be noted that the studies in which the interplay between inflammaging and frailty has been investigated are not homogenous in terms of study participants and design, tools for frailty assessment and the techniques employed to measure biomarkers (Saedi et al., 2019). However, it is also to be considered that inflammaging is a complex phenomenon always accompanied by a regulatory counterpart aimed at modulating or limiting its effects, the so-called anti-inflammaging (Franceschi et al., 2007; Morrisette-Thomas et al., 2014), and that it is not yet clear the importance and interindividual variability of such anti-inflammaging, not to mention the fact that is almost always neglected in the studies on frailty, or at least not considered together with inflammaging. To this regard, the best-known examples of inflammaging and anti-inflammaging are in fact IL-6 and its soluble receptors, as well as IL-1 family and its antagonistic receptors. In order to obtain consistent and meaningful results on the association of

such molecules with pathological conditions, including frailty, it is more than likely that anti-inflammatory partners of such molecules must be taken in consideration, too.

### 2.1. IL-6, sIL-6R alpha and gp130: the triplet of inflammation or anti-inflammation

IL-6 was discovered as a growth factor of B cells, then defined as a pro-inflammatory cytokine of innate immune system, in particular produced by monocytes/inflammatory macrophages. In early 1990 s, it became clear that besides controlling many other immune cells, IL-6 is also important in the regulation of hepatocytes, hematopoietic progenitor cells, skeleton, cardiovascular system, placenta and the nervous and endocrine systems (Kishimoto et al., 1995). In addition, IL-6 may be expressed and produced by various cellular types other than immune cells, such as fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several kinds of tumor cells (Naka et al., 2002).

The IL-6 membrane receptor complex consists of the IL-6-specific alpha receptor (IL-6Ralpha, alpha chain, gp80, CD126) and the signal transducing subunit glycoprotein 130 (gp130, beta chain, CD130). IL-6 first binds to IL-6Ralpha with low affinity. The IL-6:IL-6Ralpha complex subsequently builds a high affinity complex with gp130 molecule, likely forming a final membrane hexamer (Singh and Jois, 2018). The formation of this complete receptor complex induces the subsequent activation of intracellular signaling pathways (the so-called classical signaling), leading to IL-6-dependent gene expression and IL-6-dependent cellular responses such as proliferation, migration, expression of other molecules or metabolic changes (Heinrich et al., 2003). IL-6 cellular response tightly depends on the cell type, thus is not necessarily related to inflammation. Accordingly, IL-6 is also expressed and released by both type I and type II muscle fibers where it exerts autocrine effects, in particular, through the classical signaling it has anti-inflammatory effects, leading to the activation of different anabolic pathways, increased glucose uptake and fat oxidation, and promoting the proliferation of satellite cells (Forcina et al., 2022).

Importantly, IL-6Ralpha and gp130 proteins may be secreted and circulate in the blood upon cleavage by membrane enzymes such as sheddase family members (Rose-John et al., 2017). Thus, IL-6, sIL-6-Ralpha and the soluble (s) molecule gp130 are currently considered three key molecules able to differently mediate the states of activation/inhibition, according to their different combination as follows. Soluble IL-6 is able to activate the classical signaling only when it binds the complete membrane receptor, which is expressed only in few tissues/cell types. On the other hand, the sIL-6:IL-6Ralpha complex may activate IL-6 trans-signaling by binding the membrane-bound gp130, which is ubiquitously expressed. Noteworthy, this trans-signaling seems to be the driver of inflammatory status at systemic level. Concomitantly, elevated sgp130 may act as a decoy receptor that inhibits IL-6 classical signaling by sequestering free IL-6 into IL-6:IL-6Ralpha:sgp130 complex (Jostock et al., 2001; Garbers et al., 2011; Reeh et al., 2019; Forcina et al., 2022). Overall, this evidence indicates that the reciprocal interactions of these three molecules are crucial to determine the type of IL-6 signaling. Actually, in healthy physiological conditions, IL-6 concentrations are undetectable or very low, such as 1–6 pg/ML (depending on chronological age), while sgp130 and sIL-6Ralpha are three orders of magnitude higher (ng/ML), thus they are able to buffer the IL-6 effects at systemic level (Rose-John, 2012; Forcina et al., 2022).

Since 2000, IL-6 has also been considered as one of the most important cytokines that fuel inflammaging. In this perspective, a striking amount of data has been published indicating that IL-6 is associated with a spectrum of age-related conditions, including cardiovascular diseases, osteoporosis, osteoarthritis, sarcopenia, macular degeneration, neurodegeneration, Type 2 Diabetes (T2D), certain cancers, periodontal disease, post-operative delirium in elderly, functional decline and frailty (Ferrucci et al., 2005; Leng et al., 2005; Maggio et al., 2006; Ferrucci and Fabbri, 2018; Stenholm et al., 2011; Lin et al., 2014;

Capri et al., 2014; Sluiman et al., 2022; Costantini et al., 2023). Not unexpectedly, the condition of multimorbidity is characterized by high levels of pro-inflammatory cytokines, and the different spectrum and number of morbidities may affect the level of systemic inflammation. In fact, a recent study on the incidence, prevalence, risk factors, and fluid and imaging biomarkers of mild cognitive impairment in old people has revealed that patients in the higher multi-morbidity percentiles had significantly higher IL-6 and TNF-alpha levels compared with those in the lower multi-morbidity percentiles (St Sauver et al., 2022). Similarly, the condition of frailty, often associated with multimorbidity, is characterized by a pro-inflammatory phenotype, including high level of IL-6 (Fabbri et al., 2015; Ferreira et al., 2018; McKechnie et al., 2022). Accordingly, the frailty phenotype is characterized by biomarker patterns reflecting inflammation (*e.g.* high level of systemic IL-6) or muscle catabolism in multimorbid patients (Kochlik et al., 2023). Surprisingly, a relatively low number of papers has been focused on the IL-6 triplet and frailty status, even if a seminal study was conducted in InCHIANTI project, where many different pro- and anti-inflammatory markers were assessed, including IL-6 and sgp130, that were significantly correlated with aging, except sIL-6Ralpha (Morrisette-Thomas et al., 2014). On the other hand, a recent study on frailty and inflammatory markers has shown a significant association between the sIL-6R and frailty with worsening function supporting a potential increased risk of hip fractures (Langmann et al., 2017). These results are in agreement with a previous larger study in which IL-6Ralpha was also assessed and its increased blood level was associated with an increased risk of hip fracture in a median follow-up of 7 years (Barbour et al., 2012).

Similarly, a relatively low number of papers reports the assessment of the IL-6:sIL-6Ralpha:sgp130 triplet in age-related conditions. It is reasonable to think that most of them could be characterized by a systemic pro-inflammatory condition likely mediated by IL-6 trans-signaling, among other cytokines and mediators. Interestingly, this mechanism has been demonstrated in severe cases of Corona Virus Disease (CoViD)− 19 (Rodríguez-Hernández et al., 2022). In this context, the sIL-6:IL-6Ralpha complex may have a pleiotropic effect on all the cells expressing gp130, thus stimulating a pro-inflammatory response in different cells and tissues. A study has reported a significant increase of both sgp130 and sIL-6Ralpha in post-menopause women, even if the ratio gp130:IL-6Ralpha remained quite constant, and the different concentration between IL-6 (picograms) and sgp130 (nanograms) makes IL-6 classical signaling the most achievable (Kangas et al., 2014). In other conditions, serum concentration of sgp130 has been associated with disease severity. In particular, in patients with stable coronary artery disease (CAD), sgp130 is inversely correlated to the level of coronary damage and authors suggest the adoption of low sgp130 level as an additional indicator of coronary atherosclerosis severity (Korotaeva et al., 2018). In the same direction, a recent work has shown that sIL-6Ralpha:sgp130 levels were lower in T2D patients than controls, whereas IL-6 was high and inversely correlated with sIL-6Ralpha. Moreover, low levels of sIL-6Ralpha:sgp130 and high levels of IL-6 were found in patients with T2D or T2D plus atherosclerosis (Aparicio-Siegmund et al., 2019). sgp130 was also increased in patients with metabolic syndrome, in correlation with insulin resistance, likely as a homeostatic effect to buffer the concomitant increase of IL-6 (Zuliani et al., 2010). Therefore, it is a straight-forward hypothesis that the evaluation of the ratio between sIL-6Ralpha:sgp130 and IL-6 (physiologically from 3:1–8:1) and the level of sgp130 could help in making clearer the difference between patients with different levels of multimorbidity, as they can be a measure of the capability of the body to buffer IL-6 production.

Overall, further studies should be conducted on the complete triplet IL-6:sIL-6Ralpha:sgp130 and their combination, since high levels of sgp130 may inhibit both trans- and classic signaling as both the soluble complex sIL-6Ralpha:sgp130 and sgp130 alone are able to buffer and neutralize IL-6. Longitudinal studies should be the best experimental design to test the modification of the triplet concentration and

combination, likely suggestive of the homeostasis alteration leading to the IL-6 pro-inflammatory signaling establishment along aging process and disease onset.

As far as the strategy for counteracting IL-6 effects is concerned, a growing attention to ad hoc drugs/therapies has been emerging during the last decade. The role of IL-6 and its alpha chain receptor in some autoimmune diseases including arteritis and Rheumatoid Arthritis (RA) has been demonstrated through the successful therapeutic adoption of Tocilizumab, the humanized monoclonal antibody approved against IL-6Ralpha, as antagonist of IL-6 classical and trans-signaling (Salvarani et al., 2012; Rose-John et al., 2017; Marsal Barril et al., 2022). Some positive effects have also been observed in patients with severe CoViD-19, even if the literature is discordant, since final effects of Tocilizumab have been associated with the severity level of the disease (Ghosn et al., 2021; Jain et al., 2022).

In this view, the systemic level of IL-6 may be reduced by physical exercise also in condition of morbidity, for example in T2D (García-Hermoso et al., 2023; Papagianni et al., 2023). Interestingly, master athletes show lower levels of IL-6 and sIL-6R and higher IL-10 and IL-10/IL-6 ratio compared to age-matched untrained controls (Aguiar et al., 2020), but in this context sgp130 has not been measured.

## 2.2. IL-1 superfamily members: focus on IL-33 and its soluble receptor sST2

IL-1 is the first interleukin which was discovered in 1979 (Mizel and Farrar, 1979), even if the first information on proteins acting as endogenous pyrogenic biomolecules goes back to 1940 s and 1950 s (Menkin, 1943; Atkins, Wood, 1955). IL-1 plays a crucial role in innate immune system response and inflammation modulation, acting in fever induction and acute-phase response (Rea et al., 2018). IL-1 is currently recognized as a large family including 11 members, consisting of seven agonists (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), three antagonists (IL-1Ra, IL-36Ra, and IL-38) and one anti-inflammatory cytokine (IL-37). The IL-1 family is the prototypical example of leaderless secretory proteins, a growing list of products that are delivered extracellularly despite lacking an endoplasmic reticulum (ER)-targeting leader sequence typically required for secretion. To reach the extracellular space, leaderless proteins exploit strategies collectively referred to as unconventional secretion (Garlanda, Dinarello and Mantovani, 2013; Cavalli and Cenci, 2020). In addition to proteins with established extracellular roles (e.g., cytokines), unconventionally secreted products include proteins with still unrecognized extracellular functions (e.g., ferritin), as well as proteins with established intracellular roles whose extracellular release generates new functions, which include high mobility group protein B1 (HMGB1), the archetypal damage-associated molecular pattern (DAMP) molecule. The extracellular roles of most hitherto recognized unconventionally secreted proteins point to inflammation as a conserved functional common denominator (Cavalli and Cenci, 2020; Sitia and Rubartelli, 2020). Notably, the diverse secretory routes affording IL-1β secretion are shaped by autophagic genes and mechanisms that inhibit or stimulate IL-1β synthesis and release depending on the severity of inflammatory stimuli (Claude-Taupin et al., 2018; Cavalli and Cenci, 2020).

The highly inflammatory cytokines IL-1β and IL-1α are usually present in circulation at very low levels, and their activity is effectively regulated by several decoy receptors and soluble antagonists. Extensive investigations have demonstrated that the complex IL-1 family plays a broader role shaping and orienting innate immunity and inflammation in response to different microbial or environmental challenges (Mantovani et al., 2019).

IL-33 and its unique receptor, named suppression of tumorigenicity 2 (ST2), are attracting the interest of researchers as biomarkers of aging and age-related diseases (Schmitz et al., 2005). IL-33 and ST2 are expressed in multiple types of cells, including endothelial cells, epithelial cells, smooth muscle cells, and immune cells. Two patterns of IL-33

expression were highlighted: constitutive and induced (Guo et al., 2022). In the steady state, IL-33 is constitutively expressed as a full-length protein located in the nucleus, where it binds to chromatin via the tails of histones H2A and H2B and regulates gene expression (Roussel et al., 2008). Cellular damages or different types of stressors can enhance IL-33 expression and promote IL-33 release by immune and non-immune cells. In this context IL-33 acts as an "alarmin" (alarm signal) activating inflammatory and immune responses (Cayrol and Girard, 2014). Once full-length IL-33 is released by cells, the serine proteases cathepsin G and elastase, produced by neutrophils and mast cells, can shear full-length IL-33 to produce highly active IL-33 (Lefrançais et al., 2012). Due to structural similarity of cytokine domain and their receptors between IL-33 and IL-1, early studies have focused on IL-33 similarity to IL-1 in inflammation and immune response. However, IL-33 is a pleiotropic cytokine and its functions appeared very complex and contexts-dependent. Generally, in physiological conditions IL-33 induces Th0 cells to differentiate into Th2 cells, induces M2-like macrophage polarization, promotes the formation of neutrophil extracellular traps (NETs) in models of infection, enhances NK cell aggregation at inflammatory sites and promotes the production of inflammatory cytokines TNF-α and Interferon (IFN)-γ (reviewed by Guo et al., 2022). Paradoxically, it has been recently demonstrated in animal models that IL-33 can promote immunosuppression by inducing thymic involution-associated naive T cell dysfunction, suggesting that targeting IL-33 or ST2 could be a promising strategy to rejuvenate T cell immunity to better control severe infection (Xu et al., 2022). Overall, IL-33 can play both pro- and anti-inflammatory effects, playing dual roles in the progression of several diseases including metabolic diseases (Nesic et al., 2022), neurodegenerative diseases (Rao et al., 2022) and cancers (Liu et al., 2023). Circulating IL-33 levels were investigated as biomarkers of mortality or diseases severity. Notably, low circulating levels of IL-33 were associated with increased mortality risk in critically ill patients (Krychtiuk et al., 2018), or with large infarction volume and greater stroke severity in patients affected by acute ischemic stroke (AIS) (Qian et al., 2016). Increased IL-33 circulating levels have been observed in asthma, atopic dermatitis, multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome, whereas it seems to be protective in atherosclerosis, suggesting different roles in immune-regulated diseases (reviewed in Theoharides et al., 2015).

In 1989, ST2 was first identified by 2 independent laboratories working on growth-stimulated fibroblasts, but the function of this protein has remained unclear for several years (Werenskiold et al., 1989; Tominaga, 1989). In the following years, IL-33 was identified as the ligand of ST2. The soluble form of this receptor, sST2, is the most important antagonist of IL-33 identified so far. As a soluble receptor, sST2 binds to IL-33 to prevent ST2–IL-33 binding, consequently blocking downstream signaling and immune response. sST2 is produced mostly by alveolar cells and vessel wall cells, and to a lower extent by cardiac fibroblasts and cardiomyocytes. A few studies have uncovered the complex roles of IL-33/sST2 in physiological and pathological conditions. Several studies highlighted the prognostic relevance of sST2 in heart failure (HF). In the Framingham Heart Study, sST2 levels predicted death, incident HF, and major cardiovascular events independently from standard cardiovascular risk factors and other biomarkers (Wang et al., 2012). Studies evaluating the prognostic significance of sST2 in acute HF have confirmed that this biomarker helps refine risk stratification. A meta-analysis showed that sST2 levels measured both on admission and at discharge, predict all-cause and cardiovascular mortality (Aimo et al., 2017). Overall, sST2 is a strong predictor of outcome in HF and its prognostic value is additive to natriuretic peptides (Aimo et al., 2019). Interestingly, a huge amount of data suggested that IL-33 and sST2 have clinical relevance as prognostic biomarkers not only for HF but also for different cardiovascular diseases (CVD) (Cardellini et al., 2019; Sun et al., 2021; Li et al., 2021; Jia et al., 2023). A recent study highlighted sex and age differences in serum sST2 levels depending on CVDs (Beetler et al., 2023). Notably, elevated sST2 levels were associated with

unplanned hospital admission due to major adverse cardiovascular events (MACE) within 1 year (Chen et al., 2023). Two studies on large cohorts of patients examined serum levels of sST2 to determine whether sex-specific cutoffs would enhance determination of risk for hospitalization or death (Harmon et al., 2021; Vergaro et al., 2022).

In addition to HF and others CVD, the prognostic role of sST2 was highlighted in several others age-related acute and chronic conditions, including acute distress (Erfurt et al., 2022), stroke (Krishnamoorthy et al., 2023), systemic sclerosis (Günther et al., 2022), neurodegenerative diseases (Saresella et al., 2020; Tan et al., 2023) T2D (Caporali et al., 2012; Sabbatinelli et al., 2022), metabolic syndrome (Roy et al., 2023), cancers (Akimoto et al., 2016; Cui et al., 2015; Jou et al., 2022), chronic kidney disease (CKD) (Guo et al., 2021), hypertension (Yin et al., 2019), chronic obstructive pulmonary disease (Urban et al., 2021), and critical ill patients (Krychtiuk et al., 2018). sST2 was studied also in the context of infectious diseases, such as CoViD-19 (Zeng et al., 2020; Omland et al., 2021; Park et al., 2023) and chronic hepatitis B (Yuan et al., 2020). sST2 was also established as a prognostic biomarker of nonrelapse mortality when measured early after allogeneic hematopoietic cell transplantation (HCT) and as a biomarker of pretransplantation vulnerability (Gjærde et al., 2023). Notably, sST2 was identified as an independent predictor of total mortality in a few population studies (Pfetsch et al., 2017; Filali et al., 2021; Ip et al., 2021). Overall, sST2 is currently considered a useful biomarker mainly for HF, since it is a strong predictor of outcome in HF and its prognostic value is additive to natriuretic peptides (Aimo et al., 2019). The analytical characteristics of sST2 assay are discussed in dedicated publications (Mueller and Jaffe, 2015). However, IL-33 and sST2 meet some criteria as biomarker of aging and age-related diseases: accurate measurements are available at a reasonable cost, and the biomarkers provides information not already available from clinical assessment. sST2 has been proposed as a marker for frailty but not convincingly tested so far (Cardoso et al., 2018; Sato et al., 2022).

## 3. Markers of mitochondrial dysfunction: focus on mitokines

Beyond inflammaging, and strictly related to it, another pillar of aging is the mitochondrial dysfunction. Indeed, besides energy generation, mitochondria also mediate other fundamental cellular processes, such as apoptosis, calcium signaling, amino acid and nucleotide synthesis, and reactive oxygen species (ROS) production. It is thus clear that a dysfunction of mitochondria can play a crucial role in aging process. Notably, mitochondrial dysfunction is listed among the "antagonistic" hallmarks of aging, as it can be beneficial when present at low intensity and can turn detrimental at high intensity (López-Otín et al., 2013). In fact, it is known that mitochondrial dysfunction (in the form of production of ROS or mitochondrial DAMPs) is perceived by molecular sensors related to the inflammatory response such as NF-κB, TLR9, cGAS-Sting, NLRP3, etc. (Vitale, Salvioli and Franceschi, 2013; Zanini et al., 2023). Unmodulated inflammatory activation resulting from mitochondrial oxidative damage has been demonstrated to contribute to unsuccessful aging, as consequent overproduction of ROS quickens cellular senescence and increases the secretion of SASP components (Kim et al., 2022). This process is particularly relevant for the homeostasis of the organism, and its measurement may represent a marker of aging and frailty (Bencivenga et al., 2023), which is considered a state of increased vulnerability to develop adverse outcome in response to stress (Clegg et al., 2013). On the other side, during evolution, mitochondria have developed several pathways to limit cellular damage in response to stress (Lima et al., 2022). These stress responses are indeed beneficial for the whole organism and may promote longevity. Among these stress responses, the mitochondrial unfolded protein response (UPR$_{mt}$) is a conserved quality-control transcriptional system that maintains mitochondrial protein homeostasis through a coordinated network of chaperones and proteases (Shpilka, Haynes, 2018). The UPR$_{mt}$ promotes cell survival and lifespan extension through several metabolic adaptations,

and defects in this process have been described in several human age-related diseases, such as sarcopenia (Migliavacca et al., 2019) and Alzheimer's disease (AD) (Beck et al., 2016; Sorrentino et al., 2017). Various molecular actors of the UPR$_{mt}$ have been associated with longevity.

With these premises in mind, especially from the innovative perspective of Geroscience, it is not surprising the growing interest in research for biomarkers useful in the assessment of the degree of mitochondrial dysfunction in relation to frailty (Gonçalves et al., 2022; Guerville et al., 2019). In this context, some soluble mitochondrial stress-induced molecules, known as mitokines, have been investigated, in particular fibroblast growth factor 21 (FGF21), growth differentiation factor 15 (GDF15) and Humanin (HN) (Conte et al., 2019).

Several studies have reported an increase of GDF15 levels with aging and an association of chronically elevated levels of GDF15 with several age-related diseases, including geriatric syndromes, cardiovascular conditions and metabolic disorders (Conte et al., 2022). Although its role is still not fully understood, with evidence reporting opposite functions, there is agreement that this molecule is involved in the process of mitohormesis, representing an adaptive reaction to stress (Conte et al., 2022). According to this fascinating theory, in the mechanism of body resources allocation, this mitokine counteracts the damage resulting from systemic inflammation due to intrinsic and environmental stressors, as also demonstrated in the context of cardiovascular homeostasis (Bencivenga et al., 2023). As GDF15 is linked to lipid and energy metabolism as well as to cachexia, it is also not surprising that it has been found associated to frailty (Arauna et al., 2020; Alcazar et al., 2021), muscle waste and decreased force and poor physical function (Nakajima et al., 2019; Conte et al., 2020; Oba et al., 2020; Merchant et al., 2023).

Similar to GDF15, it is still uncertain whether FGF21 exerts a beneficial or harmful role in aging and related conditions, as its release appears to determine opposite biological effects in short- or long-term (Conte et al., 2019, 2021). Anyhow, as shown in pre-clinical studies, this mitokine is involved in the regulation of age-related thymic involution, thus allowing to speculate on its role in the immunomodulation, related to mitochondrial dysfunction. The anti-apoptosis and -inflammation activities of FGF21 are also mediated by reduced expression of the cytokines TNF-α, and IL-6 (Yu et al., 2016); furthermore, circulating FGF21 levels increase with chronological age, especially in centenarians (Conte et al., 2019). At variance with GDF15, the association with frailty and diseases is not clear. In some cases, it seems that FGF21 levels are associated with decreased muscle force (Roh et al., 2021), while in others do not contribute to identify sarcopenic patients (Picca et al., 2022); in some cases, FGF21 levels have been found decreased in the plasma of AD patients (Conte et al., 2021), while in others FGF21 has been proposed as a biomarker of mitochondrial disorders (Scholle et al., 2018). As a whole, it seems that more studies are needed to clarify the role of FGF21 as a marker of frailty and diseases. However, although their crucial role in the interplay among aging, immunity and inflammation needs to be further investigated, to date it seems that a chronic upregulation of FGF21 and GDF15 determines adverse effects that exceed beneficial ones deriving from controlled acute release.

HN can be considered an anti-inflammatory molecule, exerting a cytoprotective role through different signalling pathways, including anti-apoptotic activity (Thummasorn et al., 2018). Its protective activity against oxidative stress and inflammation has been confirmed by different evidence reporting a beneficial effect in several frailty-associated conditions, including cardiovascular diseases and neurodegenerative disorders. Interestingly, even if not fully clarified, the neuroprotective activity of HN seems to be partially due to an interference mechanism with the aforementioned inflammatory cytokines (Conte et al., 2020). While it is clear that HN is associated with healthy lifespan (Yen et al., 2020), increased HN levels are actually found in old age, likely reflecting an increased level of mitochondrial stress (Conte et al., 2019; Conte et al., 2021); quite surprisingly, no data

are available on the possible value of HN as a biomarker of frailty, even though an inverse correlation between HN and frailty could be expected.

It is still a matter of debate whether mitokines can be considered as produced exclusively in response to mitochondrial dysfunction or rather to many types of stress, including ER and lysosomal stress, therefore casting some legitimate doubts on the specificity and biological meaning of these biomarkers. Other possible markers of mitochondrial dysfunction are plasma levels of cell-free mitochondrial DNA (mtDNA). The circulating levels of mtDNA were found to increase gradually after the fifth decade of life and correlate with serum inflammatory markers such as TNF-α and IL-6 (Pinti et al., 2014), supporting a role of circulating mtDNA in age-associated low-grade inflammation. In some studies, the levels of circulating mtDNA have been associated with frailty and inflammatory markers (in particular, CRP, soluble TNF-α receptor 1 [sTNFR1], and IL-6) in old people (Jylhävä et al., 2013; Nidadavolu et al., 2023). The usefulness of circulating mtDNA (and maybe nuclear DNA) as a marker to detect/predict frailty and multimorbidity is not yet very clear, as studies are sparse and the measurement is not yet standardized, is very operator- and protocol-sensitive and finally its biological meaning is still unclear, even though a role in inflammaging has been proposed, being mtDNA among the ligands for natural immune receptors (Franceschi et al., 2017).

## 4. Markers of neurodegeneration: focus on neurofilament light chain

There is an urgent need for sensitive biomarkers that allow for early detection of neurodegenerative processes and are sensitive to disease progression (Outeiro et al., 2023). Current diagnostic criteria for Alzheimer and Parkinson disease mainly rely on the manifestation of cardinal cognitive or motor symptoms but it is now well established that neuronal dysfunction and loss begins well before the development of clinical manifestations. Early detection is going to be critical for disease modification especially in light of the registration of two monoclonal antibodies in AD by the FDA and numerous ongoing clinical trials in Parkinson's Disease (PD) (Jeremic et al., 2023). Neurofilament light chain (NfL) is a neuronal cytoplasmic protein, highly expressed in large caliber myelinated axons (Barro, Chitnis and Weiner, 2020; Khalil et al., 2018). NfL is a subunit of Neurofilaments (Nfs), proteins classified as Intermediate filaments (IF) exclusively located in the soma of neurons, which together with glial filaments are the main constituent of neuronal cells. NfL is the backbone of Nfs and their most abundant component. Also, it is the best candidate among the Nfs subunits to be measured because it is the most soluble one (Gafson et al., 2020; Herrmann and Aebi, 2016; Yuan et al., 2017). High NfL levels in biofluids, such as cerebrospinal fluid (CSF) and blood, are indicative of axonal damage and neuronal death as NfL concentration rises proportionally to the degree of axonal injury and degeneration. For these reasons, NfL is considered a valid biomarker of neurodegeneration, useful for diagnosis, prognosis and treatment response monitoring (Delaby et al., 2022; Dutta et al., 2023; Gaetani et al., 2019a; Hansson, 2021; Wang et al., 2019). In particular, its usefulness as biomarker has been investigated in many proteinopathies such as AD, Frontotemporal dementia (FTD) (Forgrave et al., 2019; Staffaroni et al., 2022), PD and synucleinopathies (Aamodt et al., 2021; Canaslan et al., 2021), Creutzfeldt-Jakob disease, Huntington's disease (HD) (Jeromin and Bowser, 2017), Amyotrophic lateral sclerosis (ALS) (Ingannato et al., 2021), Multiple sclerosis (MS) (Jeromin and Bowser, 2017). Recent studies have demonstrated that NfL levels can be used to discriminate between different preclinical stages of disease like Subjective Cognitive Decline (SCD) and Mild Cognitive Impairment (MCI) (Giacomucci et al., 2022) or traumatic brain injuries (TBI) and stroke (Heiskanen et al., 2022; Wu et al., 2022).

In testing NfL to assess cognitive decline, one must consider whether such decline results from either acute events or a chronic neurodegenerative condition (Fig. 1). In case of cognitive impairment after an acute neuronal event such as stroke (ischemic or hemorrhagic), blood NfL
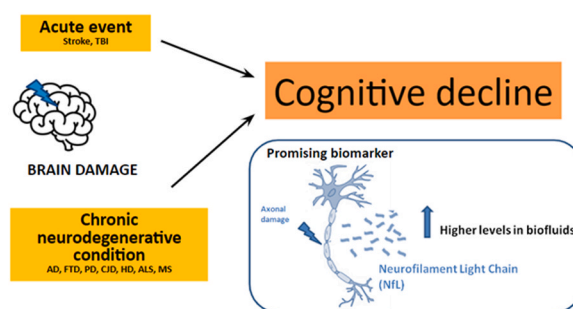


**Fig. 1.** NfL as marker for neurocognitive decline. After an axonal damage, resulting from either an acute event or a chronic neurodegenerative disorder, NfL levels increase in biofluids. Since cognitive decline follows neurodegeneration, then NfL is a promising biomarker of cognitive decline. Abbreviations: Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Creutzfeldt-Jakob disease (CJD), Frontotemporal dementia (FTD), Huntington's disease (HD), Multiple sclerosis (MS), Parkinson's disease (PD), Traumatic Brain Injury (TBI).

increases during the first days and remains elevated over 3–6 months (Gattringer et al., 2017; Shahim et al., 2016; Tiedt et al., 2018). Blood NfL levels were reported to be proportional to the lesion burden (Duering et al., 2018). At variance, during neurodegenerative disorders NfL levels increase in biofluids proportionally with neurodegeneration and cognitive impairment (Checkoway, Lundin and Kelada, 2011; Lin et al., 2019; Preische et al., 2019). Studies on AD or other forms of dementia showed that higher NfL concentration corresponded to poorer Mini Mental State Examination (MMSE) performance and was associated with the cognition domains of memory, language, attention, executive and visuospatial functions (Bos et al., 2019; Lewczuk et al., 2018; Mattsson et al., 2016; Preische et al., 2019; Rolstad et al., 2015; Steinacker et al., 2018). A correlation between NfL levels and cognition was found also in other tauopathies, such as Progressive Supranuclear Palsy and corticobasal degeneration with a deterioration in global cognition (Marchegiani et al., 2019). An inverse relationship with attention and processing speed was reported in HD (Byrne et al., 2017). Studies on patients affected by MS established an association with high NfL levels and a decreased cognition, in particular in executive function, working memory, attention control, verbal fluency, verbal episodic and semantic memory (Gaetani et al., 2019b; Kalatha et al., 2019; Mattioli et al., 2020). Contrasting results were reported for PD and its relationship with cognition, measuring specific domains of verbal fluency and perception speed (Bäckström et al., 2015; Hall et al., 2015; Mollenhauer et al., 2019). With regards to ALS, a positive relationship between the magnitude of annual MMSE score loss and NfL concentration was reported (Olsson et al., 2019), as well as a correlation with the age at onset, allowing the discrimination between bulbar or spinal onset (Ingannato et al., 2021). The most important application of NfL as a biomarker is related to the utility in preclinical stages of cognitive decline. Higher plasma NfL concentration was related to an increased risk to develop dementia (de Wolf et al., 2020). Studies reported that plasma NfL can predict AD and MS up to 6 years before clinical manifestation. Instead, in faster neurodegenerative diseases with a short pre-symptomatic stage, such as ALS, plasma NfL increases in the transition from the preclinical to clinical phase, with a fast acceleration as symptoms loom (Gaetani et al., 2021). Studies on pre-symptomatic patients showed different NfL dynamics based on type of disease and genetic mutation carried. With regard to ALS, in FUS (Fused in Sarcoma) and C9orf72 (chromosome 9 open reading frame 72) gene mutation carriers, elevated plasma NfL levels were observed 2 and 3.5 years, respectively, before ALS onset (Benatar et al., 2019). In SOD1 (superoxide dismutase 1) mutation carriers, NfL increased only 12 months before ALS onset (Benatar et al., 2018). Regarding FTD, the GENFI (Genetic Frontotemporal dementia Initiative) study showed that NfL

began to be altered 30 years prior to FTD onset in C9orf72 mutation carriers and 15 years before disease onset in those who carry a mutation in GRN (Granulin). MAPT mutation carriers showed higher NfL concentration only close to symptom onset (Staffaroni et al., 2022; Van Der Ende et al., 2019; Wilke et al., 2022). With regard to AD, in PSEN1 (Presenilin 1), PSEN2 (Presenilin 2) and APP (amyloid precursor protein) pathogenic mutation carriers, NfL could predict AD 16 years before first clinical manifestation, with plasma levels significantly higher in mutation carriers compared to non-carriers (Weston et al., 2019). Moreover, NfL can be a useful biomarker to monitor cognitive decline in preclinical stages of AD, discriminating SCD to MCI and AD. Plasma NfL levels rise in stage-dependent manner and are correlated with amyloid biomarkers in CSF (Giacomucci et al., 2022).

With regards to frailty, recently, researchers have further noted that plasma concentration of NfL is dependent on kidney function, so that interpretation of frailty in patients suffering from CKD, which can affect the clearance of proteins, should be taken with caution (Ladang et al., 2022). A recent study failed to find associations of circulating NfL and progranulin (PGRN) levels with frailty among community-dwelling older adults in adjusted analyses (Kaloostian and Shil, 2022; Lu et al., 2022). The study included 507 older adults (mean [standard deviation] age, 76.7 [4.5] years) with plasma NfL and PGRN measurements from the Multidomain Alzheimer Preventive Trial (MAPT). The time point of biomarker measurements, either 12 or 24 months after study enrollment, was defined as the baseline for each participant. Frailty phenotype (robust, pre-frail, and frail) was assessed at 12, 24, 36, 48, and 60 months by Fried's frailty criteria. The cross-sectional associations between plasma neurodegenerative biomarkers and frailty severity were examined using logistic regressions. According to authors, whether plasma neurodegenerative markers serve as potential biomarkers of frailty requires further investigation.

With regards to Multimorbidity, NfL levels are influenced by aging-related medical conditions. In normal physiological conditions, low quantities of NfL are constantly released in interstitial fluid, increasing linearly with aging. NfL and age show a positive association. Compared to 20-years old subjects NfL levels are twofold and sixfold higher in 50-years old and 80-years old individuals, respectively (Gaetani et al., 2019a). Aging comorbidities such as multiple cardiovascular conditions (atrial fibrillation, myocardial infarction), psychiatric conditions (depression and anxiety), T2D, CKD, could explain some of the age associations. In a study conducted on a small-scale sample of septuagenarians in Gothenburg, plasma NfL was seen to be associated with a smaller hippocampal volume and larger ventricular volumes, detecting aging pathophysiological changes (Dittrich et al., 2022). Meeker et al. (2022) reported a statistically significant correlation between higher NfL levels and greater white matter hyperintensity volume (WMH), reflecting aging and brain matter damage. NfL concentration can also be influenced by body-mass-index (BMI), T2D, and hypertension (Barro et al., 2020). In particular, a higher BMI was correlated with lower NfL in plasma. A positive correlation was found with cardiovascular conditions (Polymeris et al., 2020). Among cognitively unimpaired individuals, aged 51–95 years, NfL correlated with age and comorbidity variables, in particular higher plasma levels were related to higher Charlson Comorbidity Index and CKD (Syrjanen et al., 2022). CKD is characterized by a low glomerular filtration rate and, in consequence, by a reduced clearance of proteins in the blood. Indeed, CKD was associated with a higher plasma NfL concentration (Xu et al., 2021). In the normal aging population, NfL correlated with cystatin C concentration, estimated glomerular filtration rate, and comorbidities such as cardiovascular diseases, neurological disorders or history of fracture (Ladang et al., 2022), however, the impact of other variables, such as plasma protein composition, blood cell counts, liver and renal clearance, presence of comorbidities and therapies was not investigated (Palermo et al., 2020).

## 5. Next generation biomarkers i.e. extracellular vesicles

Extracellular vesicles (EVs) comprise a heterogeneous population of vesicles (sized 50–1000 nm) that originate from cellular membrane budding in both physiological and pathological conditions. EVs are pivotal mediators of intercellular communication that influence coagulation, inflammation, endothelial function, and angiogenesis (Liu and Wang, 2023). Many cell types release EVs that have been isolated in peripheral and cord blood, urine, saliva, cerebrospinal fluid, sputum, bronchoalveolar lavage fluid, atherosclerotic plaques, ascites, postoperative drainage fluid, chyloid fluid, vitreous eye liquid, and synovial liquid (Liu and Wang, 2023). Under homeostatic conditions, constitutive ectocytosis leads to a permanent process of vesiculation. EV shedding is influenced by hormones (e.g. progesterone, estrogen, insulin), fatty acids, ROS (e.g. hydrogen peroxide), and by the overall cell status: for example, both stimuli that cause programmed cell death via apoptosis and those leading to survival by autophagic induction influence the extent of EV generation and the characteristics of the EVs that are released.

EVs retain antigens from their parental cells. These molecules influence EV uptake by target cells as well as target cell behaviour. EVs carry diverse types of cargo, including proteins, bioactive lipids, and nucleic acids (coding and noncoding RNAs, DNA fragments) that can be transferred to target cells (Turchinovich, Drapkina and Tonevitsky, 2019). Recent studies showed that EVs carry diverse types of RNA, including mRNAs, microRNAs (miRNA), small interfering RNAs, long non-coding RNAs, transfer RNAs, piwi-interacting RNAs, small nucleolar RNAs, etc., all of which may play relevant functional roles in recipient cells (Lunavat et al., 2015; O'Brien et al., 2020). In particular, EVs play a role in the horizontal transfer of genetic information, since EV-borne mRNAs and miRNA can be translated and modify gene expression in target cells (Valadi et al., 2007). Additionally, EVs may contain DNA molecules such as mtDNA and single- and double-stranded DNA fragments (Guescini et al., 2010; Cai et al., 2013; Manni et al., 2023).

In the blood, platelet-derived EVs are usually more abundant than those released by monocytes, other leukocytes, endothelial cells, and erythrocytes (Liu and Wang, 2023), possibly reflecting the role of platelets as guardians of endothelial integrity. For example, platelet-derived EVs accumulate in systemic sclerosis, a condition characterized by early and persistent activation and damage of the endothelial cells within the microcirculation, and reproduce microvascular damage and fibrosis when injected in experimental animals (Manfredi et al., 2022; Maugeri et al., 2018). The total detectable amount of circulating EVs is determined by their formation and clearance rates. Clearance mechanisms are manifold and most have not been fully elucidated yet, but this field is gaining increasing attention: a role for opsonins in regulating the fate of vesicles released systemically as a consequence of localized tissue damage has been advocated (Zhou et al., 2018). Flow cytometry is the gold standard method to study EVs in association with Western blotting and proteomic approaches to evaluate their cargo. It can be envisaged that EVs can become useful biomarkers for both senescence or specific age-associated conditions, as briefly summarized below. Interestingly, the best characterized moiety involved in EV clearance, MFGE8 (lactadherin) has been implicated in the sensitivity to coronary atherosclerosis on the one hand (Ruotsalainen et al., 2022), and on the other, the medin amyloid, consisting of a fragment of MFGE8, is found in the aging human vasculature and in patients with AD within vascular amyloid-β deposits with MFGE8 expression levels that are associated with cognitive decline (Wagner et al., 2022).

### 5.1. EVs in aging

Cell senescence is associated with increased release of EVs carrying specific cargos (Urbanelli et al., 2016), including miRNAs. For example, the expression of miR-146a, miR-21 and miR-223 is higher in EVs

derived from senescent cells that accumulate in the plasma of old mice (Alibhai et al., 2020). The generation of EVs may have evolved as a strategy to eliminate unnecessary components from cells undergoing senescence. Whatever their teleology, senescent EVs have distinct biochemical contents and can transmit senescence signals to neighboring cells and tissues, promoting organismal aging and the pathogenesis of age-related diseases (Franceschi et al., 2017). Riquelme et al. (2020) induced senescence in human umbilical vascular endothelial cells (HUVECs) and observed increased production of small EVs by senescent cells. A greater release of small EVs enriched in miR-21–5p and miR-217 from senescent HUVEC cells was also reported and shown to induce epigenetic and pro-senescent changes in recipient cells (Mensà et al., 2020).

Aging has been associated with specific miRNA profiles in EVs in several body fluids, including serum, plasma, follicular fluid, and saliva. Alibhai et al. (Alibhai et al., 2020) found several miRNAs to be differentially expressed in circulating EVs in young and old mice. Specifically, miR-146a, miR-21, miR-22, miR-223, miR-145, and let-7a were found to be increased in EVs from old animals, while miR-212 and miR-455 exhibited higher expression in young EVs. Similarly, in small EVs collected from the plasma of healthy subjects aged 40–100 years the content of miR-21–5p was age-dependent (Mensà et al., 2020). CD81 + small EVs displayed lower levels of miR-16–5p, miR-214–3p, and miR-449a, but higher levels of miR-125b, miR-155–5p, and miR-372 in human follicular fluid from aged individuals (Battaglia et al., 2020). EVs miR-24–3p was found to increase with aging in human saliva (Machida et al., 2015). Other nucleic acid molecules such as mtDNA and telomere fragments are present in circulating EVs (Lazo et al., 2021; Lanna et al., 2022). At least for mtDNA, their levels have been found to be influenced by age (Lazo et al., 2021), while it is not yet clear whether also telomere fragment levels may change according to the age of the donors.

EVs can also carry mRNAs encoding pro-inflammatory cytokines, and their quantities were demonstrated to be increased with aging in humans and in EVs derived from macrophages exposed to pro-inflammatory stimulants (Mitsuhashi et al., 2013; Shah, Patel and Freedman, 2018; Lananna and Imai, 2021). Interestingly, EVs can both mitigate and aggravate inflammatory conditions. Exosomes derived from miR-140–5p-overexpressing human synovial mesenchymal stem cells limited osteoarthritic damage and induced cartilage regeneration in rats (Tao et al., 2017). Furthermore, EVs derived from endothelial progenitor cells and adipose stem cells have pro-angiogenic and anti-apoptotic effects (Deregibus et al., 2007; Lai et al., 2010; Cavallari et al., 2017), mediated by the delivery of specific cargo, including mRNA and miRNAs, which activate signaling pathways involved in angiogenesis and tissue repair. For instance, miR-214, known to control endothelial cell function and angiogenesis, plays a crucial role in exosome-mediated signaling between endothelial cells (van Balkom et al., 2013). Cardiac progenitor cell-derived exosomal miRNAs were shown to play crucial roles protecting the myocardium from oxidative stress (Xiao et al., 2016). EVs can also target the brain and contribute to neurological diseases (Williams et al., 2019; Clement and Omar, 2012).

Additional evidence implicates EVs in aging and age-related diseases. For example, the transfer of senescence-associated miRNAs though EVs promotes the loss of bone marrow stem cells and adipogenesis (Shouzu et al., 2000), thus possibly favouring the development of myelodysplasia. EVs are involved also in the impairment of bone formation (Davis et al., 2017) and osteoporosis and promote sarcopenia by restricting muscle satellite cells' function (Dai et al., 2022). EVs contribute to atherosclerosis by increasing vascular calcifications and higher levels of platelet-, erythrocyte- and leucocyte-derived EVs were found in old people with cognitive impairment compared to age-matched controls with normal cognitive performance (A Magalhães et al., 2019). In general, platelet- and erythrocyte-derived EVs accumulate in older individuals. It has been postulated that this increase may be underpinned by the remodelling of cell membranes related to the aging process (Urbanelli et al., 2016), but defects in their clearance might contribute as well (see above). Endothelial EVs do not apparently accumulate in old people but their procoagulant activity under stress conditions is higher than in younger individuals (Karlaftis et al., 2014).

The unique cargo carried by circulating EVs during aging and disease progression offers a great opportunity to use EVs and their content as non-invasive markers of aging (Kalluri and LeBleu, 2020; Prattichizzo et al., 2019). In addition, the observation that changes in miRNA content in aged individuals can be partially rescued with senolytic treatment (Jeon et al., 2019; Alibhai et al., 2020), combined with the emerging causal role played by cell senescence in aging, raises confidence that EVs might soon serve as valuable tools also to monitor individual responses to aging-related therapeutic interventions.

## 5.2. EVs in frailty

The number of total EVs was fond not to differ significantly between frail and non-frail individuals. However, frail people had an increased concentration of phosphatidylserine-exposing EVs compared with non-frail older adults (Arauna et al., 2021). Since exposed phosphatidylserine is a typical eat-me signal rapidly eliminated by tissue resident phagocytes, those EVs may result from defective clearance. The role of EV phosphatidylserine is unclear, however, it may be involved in promoting atherothrombosis, a condition that characterizes frailty (Alonso-Bouzón et al., 2014). Of interest, EVs in the blood of frail patients even in pre-geriatric age are enriched in endogenous intracellular moieties associated with inflammaging, in particular mtDNA, which is associated with chemokines and other inflammatory proteins (Byappanahalli et al., 2023). Strikingly, the economic conditions of the patients, poverty in particular, affects the inflammatory cargo contained within EVs (Byappanahalli et al., 2023). EVs derived from microglia accumulate in the plasma of frail patients with cognitive impairment, and might directly cause neurotoxicity (Visconte et al., 2023). Moreover, EVs and cell-free mtDNA accumulate in the plasma of old adults living with HIV and are both associated with cognitive dysfunction (Johnston et al., 2023), further connecting the above observations.

## 5.3. EVs in obesity and diabetes

In age-associated conditions such as obesity, insulin resistance, and diabetes, EVs can serve as biomarkers and regulators of metabolic processes (Pardo et al., 2018; Lananna and Imai, 2021). The adipose tissue is a *bona fide* endocrine organ that influences energy balance, glucose metabolism, vascular and immune functions also via adipokines and cytokines incapsulated in EVs (Kranendonk et al., 2014). EVs are generated from adipocytes and adipose tissue macrophages and are increased in obese subjects (Kranendonk et al., 2014). Endothelium-, platelet- and leucocyte-derived EVs are elevated too, and possibly determine the hypercoagulation state typical of obesity (Campello et al., 2015). However, the majority of circulating miRNAs transported by EVs apparently derive from adipocytes, suggesting that the majority of circulating EVs are of adipocytic origin (Thomou et al., 2017). In T2D, a condition typically associated with obesity, a higher EV concentration was observed as compared with healthy controls, and insulin resistance measured through the HOMA-IR resulted significantly associated with EV concentration (Freeman et al., 2018). EVs connect the adipose tissue with other organs, such as the lung, playing an instructive role in asthma associated to obesity and in cachexia caused by lung cancer (Miethe et al., 2023). Of interest, adding to complexity, both detrimental and protective effects of adipose tissue-derived EVs on the asthmatic lung have been described (Miethe et al., 2023).

## 5.4. EVs in cardiovascular diseases

It has been demonstrated that EVs are augmented in patients suffering from hypertension and cardiovascular diseases (Shantsila

et al., 2010). Furthermore, EVs were found elevated also in first-degree relatives of patients with premature coronary artery disease, whereas people without a positive family anamnesis did not have elevated levels of EVs (Bulut et al., 2009). EVs released by the adipose tissue (see above) are apparently preferentially taken up by the heart, possibly justifying the plethora of cardiovascular effects in which they are involved, such as diabetic cardiomyopathy, facilitated heart ischemia, pressure overload (Michel, 2023). EVs levels correlate with the degree of endothelial dysfunction, which is an early alteration that precedes atheroma formation (Shantsila et al., 2010). Moreover, EVs correlate with the degree of cardiovascular disease severity (Shantsila et al., 2010) and their accumulation correlates with intima-media carotid artery thickness (Chironi et al., 2010).

EVs and their cargo might thus play an active role in driving the inflammation which underpin the development and progression of atherosclerosis (Collura et al., 2020), and considering their association with both early and advanced phases of cardiovascular disease, they could become a biomarker of plaque vulnerability and a predictor of future major ischemic events. Conversely, the cardiovascular protection associated to regular physical exercise on the visceral accumulation of lipids, atherosclerosis progression etc. might be related to the biological action at distant sites of EVs of skeletal muscle origins (Wang et al., 2023).

## 6. Next generation biomarkers ii. DNA methylation and epigenetic clocks

Epigenetic variation, an umbrella term encompassing reversible changes in the genome, is located at the interface between genes and environment and includes chemical changes at specific sites in the genome, that represent the result of gene-by-environment interactions (Hüls and Czamara, 2020; Nabais et al., 2023). Among epigenetic modifications, DNA methylation (DNAm) represents by far one of the most investigated variations. Indeed, there are millions of sites in the genome – called CpG dinucleotides – where the addition of a methyl group can affect the transcriptional regulation of one or more genes nearby, especially when it occurs at the level of promoters, enhancers and other important genomic regions (Reale et al., 2022). Such CpG sites can be methylated at different levels across cell types and individuals, representing a natural source of epigenetic variation. This variation is typically measured as the proportion of cells in a tissue that are methylated at a given CpG site, the so called CpG methylation fraction (or state) (Li, Koch and Ideker, 2022). This fraction is frequently assessed through techniques such as pyrosequencing or methylation microarrays. Pyrosequencing detects the methylation levels of individual CpG sites in a small polymerase chain reaction (PCR) product (usually 150–200 bp) obtained with primers common to methylated and unmethylated sequences after bisulfite conversion (Poulin et al., 2018). C and T nucleotides at individual sites are converted to amounts of released pyrophosphates by the primer extension method, and their amounts are accurately quantified bioluminometrically with a Pyrosequencer system (Li, 2021). Methylation arrays enable quantitative interrogation of selected methylation sites across the genome, offering high-throughput capabilities that minimize the cost per sample. These arrays contain collections of different oligonucleotides fixed on a solid substrate that can hybridize to complementary DNA strands (Campagna et al., 2021). Of note, the use of methylation arrays has spread more and more in the last two decades to investigate the epigenetic underpinnings of many complex disorders and traits, in Methylation (or Epigenome) Wide Association Studies (MWAS, or EWAS) (Campagna et al., 2021), which helped clarifying at least in part the epigenetic basis of many complex disorders. However, there are other, and potentially more powerful predictors of disease risk based on the use of epigenetic data resulting from methylation arrays, like methylation scores and epigenetic clocks. The former are computed as the weighted sum of the product of each CpG's methylation level by the epigenetic association of that CpG with

the same phenotype, taken from an independent (training) MWAS, and can be conceived as sort of polygenic scores built upon epigenetic rather than genetic variation (Hüls and Czamara, 2020). However, to the best of our knowledge, the use of these tools is still scarce and prevalently limited to a low number of prevalent health conditions, lifestyles and mortality so far (Nabais et al., 2023; Yousefi et al., 2022; Huan et al., 2022).

DNA methylation age estimators – better known as epigenetic clocks - represent instead an increasingly used tool for the prediction of many complex disorders (Gensous et al., 2017). These tools are based on the existence of a very well-known epigenetic phenomenon, namely that many CpG sites across the genome show a consistent hypo- or hyper-methylation trend as the organism ages. This property makes possible to estimate the age of an individual based on the degree of methylation of these CpGs, with a relatively high accuracy, exploiting machine learning algorithms like penalized and Elastic Net regressions (Li, Koch and Ideker, 2022; Yang et al., 2023), but also through deep learning models (Galkin et al., 2021). Using these algorithms, more than twenty epigenetic clocks have been proposed in the recent years, which can be divided into three generations of clocks, based on the rationale at their bases (Bergsma and Rogaeva, 2020). First generation (chronological) clocks – like the pioneering Hannum and Horvath clocks - are aimed at estimating chronological age as accurately as possible (Horvath et al., 2013; Hannum et al., 2013), while second generation (biological) clocks – like the most recent DNAm PhenoAge and GrimAge - are built to accurately predict aging-related phenotypes and risks, like mortality (Levine et al., 2018; Lu et al., 2019). Both types of clocks return a DNAm age estimation – i.e. an index of the actual underlying age of an organism – which is fundamental to compute DNAm aging, defined as the discrepancy (or difference) between biological and chronological age of an organism (the higher the discrepancy, the more accelerated is the biological aging). This discrepancy has proven to be a useful predictor of many clinical risks, such as mortality for all and specific (mostly cancer and cardiovascular) causes (Christiansen et al., 2016; Perna et al., 2016; Jylhävä et al., 2019; Marioni et al., 2018), healthy aging markers like walking speed, cognitive performance and other frailty measures (McCrory et al., 2021). Finally, third generation clocks are conceived to compute the rate of biological aging – or aging pace – in an organism, based on repeated longitudinal epigenetic measures, like DunedinPoAm (Belsky et al., 2020), and DunedinPACE (Belsky et al., 2022). These clocks – which should be interpreted as years of biological aging per year of chronological aging (with values > 1 suggesting an accelerated pace of aging), well predict all-cause mortality risk, morbidity, disability and aging-related decline, sometimes even independently on other epigenetic clocks (Belsky et al., 2020; Belsky et al., 2022).

### 6.1. Epigenetic clocks as markers of neurodegenerative disorders

In spite of their very good performance as biological aging and age-related clinical risk predictors (see above), epigenetic clocks have been relatively scarcely investigated for an association with neurodegenerative disorders, with partly contrasting and partly concordant findings (see Yang et al., 2023, for a comprehensive review). Blood DNAm clocks have been initially suggested as biomarkers of both dementia (of which AD represents 70% of all cases) and Mild Cognitive Impairment. However, associations were not always robust and consistent across studies (Fransquet et al., 2018; Zhou et al., 2022), in spite of some suggestive evidence that accelerated aging was associated with worse cognitive performance (Zhou et al., 2022). More recently, third generation clocks were reported to be associated with both dementia/MCI and cognitive tests commonly used to screen for these disorders (Sugden et al., 2022), suggesting that these clocks may be more useful to predict cognitive decline progression. Moreover, current evidence suggests that epigenetic clocks trained on brain tissues show a better performance than their blood-based counterparts in predicting AD endophenotypes like cognitive decline (Sugden et al., 2022; Levine et al., 2015) and related

neuropathological traits, including β-amyloid load, tau protein and Lewy body pathology (Levine et al., 2015; Grodstein et al., 2021). Recently, a novel brain age predictor (PCBrainAge) based on the application of principal component analysis and regularized regression to methylation data from different brain regions has been demonstrated to be more strongly associated with clinical AD dementia, pathologic AD, and APOE ε4 (risk allele) carrier status, than previous epigenetic age predictors (Thrush et al., 2022). This may be explained by different reasons, such as brain's unique methylation profile, heterogeneity across specialized neuronal and glial cell types, and distinct developmental patterns, suggesting PCBrainAge as a promising tool to predict brain aging patterns, AD risk and resilience (Thrush et al., 2022).

As for PD, the evidence supporting a potential application of DNAm clocks in its risk prediction is limited to blood-based markers and based on a very scarce quantity of manuscripts published in the field. A case-control analysis reported PD patients to have a higher DNAm age based on different epigenetic clocks (Horvath, Ritz, 2015; Paul et al., 2021), some of which were also associated with a faster cognitive decline and motor symptoms progression within patients (Paul et al., 2021). However, this association was not replicated in another longitudinal PD patient cohort (Tang et al., 2022), where only a strong positive association between Horvath DNAm age and an increased incident risk of PD was observed, along with an inverse association with age-at-onset. This finding was in line with two PD case-control studies analyzing different epigenetic age acceleration markers, including Horvath's (Horvath, Ritz, 2015) and DNAm PhenoAge clocks (Levine et al., 2018).

Overall, although these associations look promising, evidence available makes it difficult to establish epigenetic clocks as robust predictors of neurodegenerative risk in the general population, for many reasons. First, the scarcity of studies available, which is especially pronounced for PD. Second, the prevalent use of blood-based DNAm age markers, which likely suffer from a notable discrepancy from typical brain methylation patterns. Third, the heterogeneity of study designs and in particular the case-control setting of many studies, which do not allow to establish clear directions of effects, nor to rule out potential reverse causality biases between neurodegeneration and epigenetic aging. In the future, these limitations may be overcome through two key steps. First, further studies in longitudinal population cohorts are warranted to establish the accuracy of such aging clocks in predicting incident neurodegenerative risk, onset and progression. Second, the spread of in silico tools to interpret findings made on blood DNAm levels in the landscape of brain cells (*e.g.* the Blood-Brain Epigenetic Concordance platform) (Edgar et al., 2017) may allow us to translate blood-based observations and prediction on brain tissues, so to make epigenetic tools for clinical risk prediction useful and effective also for neurodegenerative and other central nervous system disorders.

## 7. Next generation biomarkers iii. Gut microbiota as potential marker for osteoporosis and sarcopenia

Osteoporosis (OP) and sarcopenia are two conditions strictly related to aging and frailty. Both OP and sarcopenia are complex phenomena whose diagnosis is usually posed with imaging or functional tests but not circulating biomarkers, despite the fact that some of them are used (or proposed) for clinical screening, such as Procollagen type 1 N-terminal propeptide, total or bone-specific alkaline phosphatase, C-terminal telopeptides of Type I collagen, C-terminal Agrin Fragment. Emerging evidence indicates that gut microbiota (GM) modifications are a potential contributing cause for these conditions, and there are many studies indicating that some of such modifications are age-dependent (Collino et al., 2013; Biagi et al., 2016). Therefore, it is interesting to consider the possibility that the abundance of specific microbial strains can represent not only a sign of aging but also a risk factor for the development of OP and sarcopenia and thus, *sensu lato*, a biomarker for these conditions.

OP is considered the most frequent metabolic skeletal disorder and is characterized by increased skeletal fragility owing to a decrease in bone quantity and/or quality and a higher risk of fractures (Lu et al., 2021; Ohlsson and Sjögren, 2015; Aspray and Hill, 2019). Fractures due to osteoporosis represent a significant health concern and lead to a considerable economic burden on healthcare systems (Ohlsson and Sjögren, 2015). It is known that different inflammatory disorders are associated with osteoporosis and that high levels of inflammatory markers are associated with a low bone mineral density, high bone resorption, bone loss, and augmented fracture risk (Steves et al., 2016). Interestingly, these inflammatory markers are influenced by alterations in GM (Steves et al., 2016). Since GM is easily malleable, this might have an important therapeutic impact (Steves et al., 2016). In particular, the relevance of GM on bone mass in health and disease has been explored (Ohlsson and Sjögren, 2015) and it has been suggested that alterations in GM could represent a potential novel biomarker of bone metabolic activity and a potential novel therapeutic target for osteoporosis and fracture prevention (Hernandez et al., 2016; Ohlsson and Sjögren, 2015). Moreover, GM represents a biological gateway among bone health and external environment as well as the bridge that connects the skeleton with different other systems, such as the digestive system, the immune system, and the endocrine system (Lu et al., 2021).

GM can affect skeletal homeostasis by influencing host metabolism, immunity, hormone secretion, and gut-brain axis (Behera et al., 2020). Interestingly, it has been suggested that GM can stimulate bone formation by affecting the intestinal metabolism of short-chain fatty acids (SCFA) (Behera et al., 2020). Clarifying the specific regulatory effects of GM on bone homeostasis and identifying the mechanisms involved via metagenomics and metabolomics approaches could be an interesting area of research (Lu et al., 2021). In addition to probiotics, prebiotics and fecal microbiota transplantation (already used in clinic), another possible therapeutic strategy, possibly safer and more effective, could be the administration of different beneficial bacteria according to the pathology and profile of the individual's GM (Lu et al., 2021).

Of note, the discovery and isolation of bacterial strains or metabolites that exert positive effects on bone and the formulation of individualized bacteriotherapy based on the different pathological characteristics could represent a promising therapeutic approach for osteoporosis treatment (Lu et al., 2021).

Recent studies have focused on the potential link between GM composition and the risk of fracture associated with OP. In particular, Ozaki et al. have studied the relationship between GM composition and osteoporosis/fracture risk in postmenopausal Japanese women (Ozaki et al., 2021). Interestingly, they have shown that gut bacteria can influence the bone mineral density and the risk of fracture (Ozaki et al., 2021). The results from this study suggest that the abundance of *Bacteroides* and *Lachnospiraceae* could have a positive effect on bone metabolism and fracture risk, whereas *Rikenellaceae* may have a negative effect (Ozaki et al., 2021). Moreover, Liu et al. have analyzed the serum levels of a gut microbial metabolite named Trimethylamine N-Oxide (TMAO) in postmenopausal Chinese women with hip fracture (Liu et al., 2020). Of note, the authors have shown that higher TMAO serum levels were associated with high risk of hip fracture, thus indicating that the rise of TMAO could play a role in osteoporosis and fracture in postmenopausal women (Liu et al., 2020). Further studies aimed to analyze the potential role of specific GM species in the increase of bone strength and in the prevention of fractures are needed to discover novel potential approaches for OP and fracture prevention (Ozaki et al., 2021). Moreover, additional studies are warranted to evaluate the potential of TMAO as a novel therapeutic target for OP and fracture (Liu et al., 2020). In addition, studies aimed to compare the intestinal microbiota composition in healthy subjects and patients with OP, with and without fractures, could identify microbiota alterations predisposing to bone loss and fracture, and can tell us whether microbiota sequencing can represent a potential novel biomarker for OP (Pacifici, 2018).

Sarcopenia refers to the age-related loss of skeletal muscle mass, strength, and function (Beaudart et al., 2014). It is a complex condition

influenced by various factors, including lifestyle, nutrition, hormonal changes, and physical activity. There is convincing evidence that sarcopenic subjects have higher risk of falls, fractures and mortality (Wong et al., 2019). While the exact mechanisms underlying sarcopenia are still being explored, emerging research suggests that GM may play a role in its development and progression (Liu et al., 2021). Several studies have investigated the association between GM and sarcopenia and frailty. A change in GM diversity was observed in sarcopenic and frail subjects, in particular species like *Firmicutes*, *Akkermansia*, *Ruminococcus* and *F. prausnitzi* appeared decreased. In general, bacterial species involved in the production of SCFA and in blunting inflammation seem to be protective against sarcopenia and frailty, while an increase in dysbiotic species, lipopolysaccharide and peptidoglycan seem to be detrimental (reviewed in Chew et al., 2023). SCFA indeed play a role in energy metabolism and muscle function (Fan and Pedersen, 2021) and can rescue muscle mass and strength in germ-free mice (Lahiri et al., 2019). Moreover, changes in GM composition, such as an increase in pathogenic or pro-inflammatory bacteria may contribute to inflammaging (O'Toole and Jeffery, 2015), and inflammation is a known contributor to muscle wasting and loss of muscle function (Ticinesi et al., 2019). On the other side, dietary intervention-mediated modifications of GM are associated with improvement of frailty and inflammation (Ghosh et al., 2020). Further mechanisms linking GM and skeletal muscle may be present and affect muscle protein synthesis, as some GM bacteria produce metabolites that can affect muscle protein synthesis pathways and potentially impact on muscle mass. In particular, GM species can produce metabolites like imidazole propionate (a trigger for insulin resistance) (Koh et al., 2018), indoxyl sulfate (involved in muscle atrophy) (Enoki et al., 2016), and urolithin A, an enhancer of mitophagy in skeletal muscle (Ryu et al., 2016). As the analysis of GM composition is not yet on the current clinical practice for neither OP, sarcopenia nor frailty, owing to the complexity of methodologies and absence of standardized and accepted signatures of bacterial species, it is proposed that these metabolites or other bacterial products may be used as surrogate biomarkers for a "good" or "bad" GM composition in terms of their effects on OP, sarcopenia and frailty and can add to the current biomarkers for these conditions with the additional advantage of providing information about possible therapeutic targets. Future studies are needed to fully understand the complex interplay between GM, inflammation, nutrient metabolism, and muscle health. The manipulation of GM through dietary interventions or the use of probiotics and prebiotics or via fecal transplantation holds promise as a potential strategy to mitigate OP or sarcopenia, but more research is required to determine the effectiveness of such approaches.

## 8. Artificial intelligence as a new perspective for aging research

The analysis and the optimal management of huge amounts of data generated by high-throughput techniques require adequate computational approaches such as machine learning (ML) and deep learning (DL) techniques – the principal components of artificial intelligence (AI) (Reel et al., 2021). Namely, ML is the capacity of systems to learn from problem-specific training data to automate the model building process and to solve related tasks, while DL is an ML concept based on artificial neural networks (Janiesch, Zschech and Heinrich, 2021). The most used AI methods can be classified in three main groups according to the underlying aims: i) understanding and learning based on the available information, ii) discovering new information, and iii) reasoning on the available information to extract conclusions (Contreras and Vehi, 2018). The first group is probably the most common one, as AI methods are usually employed to learn on data (by training and validating the data) and the main modalities to acquire knowledge are supervised, unsupervised, and reinforcement learning (Lan et al., 2018; Sidey-Gibbons and Sidey-Gibbons, 2019). Among the most important techniques there are: ML/DL, artificial neural networks, support vector machines, random forest, regression algorithms and decision trees. None of these methods is

superior, as their performance is strongly related to the quantity and quality of the data. As far as the second group (knowledge discovery), the main aim is to create algorithms to retrieve valid and potentially useful information from the data. The overall process usually requires six steps (i. e., business understanding, data understanding, preparation, modeling, model evaluation, final deployment). Among these, the 'data modeling' step is probably the most critical and technical, as the applied techniques originate from statistics and learning algorithms, aimed at detecting anomalies, identifying dependencies between variables, applying regressions, clustering, and classifications to the data. The more relevant techniques are k-means, k-nearest neighbor algorithm, hierarchical clustering, and principal component analysis. Regarding the third group (reasoning from knowledge), the ultimate goal is to create inferences in a robust way, involving the use of logical techniques such as deduction and induction, to generate conclusions from the available knowledge. The core part is the direct comparison between the knowledge base and the new obtained information. Overall, this process facilitates reasoning and helps to build new solutions based on previous cases, or to deal with ambiguous concepts and uncertainty. Representative techniques include: rule-based/case-based reasoning and fuzzy logic.

AI has revolutionized medical technologies and considering its ability to deal with complex problems in areas with a huge amount of data, one can expect promising results also in the field of aging and longevity. In the review by Zhavoronkov et al. (2019), advances and opportunities offered by AI for aging biomarkers development and anti-aging drugs discovery are overviewed. Despite AI-specific technical requirements, the computational methods used for these tasks can be integrated within the workflow to optimize several steps of aging research – ranging from the identification of aging biomarkers (e.g., imaging, 'omics', multi-modal, epigenetic biomarkers) and potential pharmaceutical targets, to the generation of ad hoc molecules with desired properties, but also of synthetic data by producing new data when patient-specific datasets are scarce (Zhavoronkov et al., 2019). In the next paragraph, the potential role of AI in the discovery and/or validation of biomarkers in the context of multimorbidity and frailty will be briefly discussed (Vetrano et al., 2019).

### 8.1. AI role in multimorbidity

Multimorbidity has a high prevalence worldwide, involving more than half of the population over 60 years (Chowdhury et al., 2023); however, currently there are no guidelines to address multimorbidity problems in terms of patient-centered solutions to predict specific outcomes and determining personalized treatments (Muth et al., 2018). This is due to the fact that no methodological framework has been developed that adequately manages the complexity of multimorbidity. A potential solution to this challenge involves the application of AI methods, which can be an alternative approach to better tackle the complex temporal dynamics of multiple interactions that characterize multimorbidity. A recent systematic review summarized the capability of ML and explainable AI techniques in predicting multimorbidity (Alsaleh et al., 2023). In particular, diabetes, hypertension, and depression were among the ten most prevalent comorbidities associated with the risk of morbidity (Alsaleh et al., 2023). The authors also identified some shortcomings related to the application of AI, the most relevant being the different phenotype categorization methods that hampered studies' comparability. By contrast, a great potential of AI may lie in its ability to capture dynamic changes over time; in this regard, a 12-year longitudinal study, by applying an ML approach (i.e., the fuzzy c-means cluster analysis), was able to track over time the clinical trajectories of older adults with multimorbidity, despite their great dynamism and complexity (Vetrano et al., 2020). In line with these observations, Table 1 summarizes potential advantages as well as disadvantages of using AI methodologies in this context.

A crucial advantage that makes the AI-research technologies favorable for studying multimorbidity over classical statistical methods, is the

**Table 1**

The pros and cons of using AI-approaches in the research field of multimorbidity (Flores et al., 2023; Majnarić et al., 2021).

| PROS | CONS |
| --- | --- |
| 1. Managing heterogeneous data in terms of complexity and diversity | - Availability of large and high-quality datasets to test and train the model |
| 1. Linking and integrating data of multiple types and sources (e.g., 'omic, clinical and non-strictly medical data) | - Complete datasets without missing observations |
| 1. Allowing for 'hidden knowledge' (the black box concept) to be extracted from data | - Enough time for model generation (training and testing phase) |
| 1. To capture the real-world scenario through the analyses of real-world data | - Limited transferability of the results in the clinical practice |
| 1. Allow to move from 'disease-only' to 'multimodal' phenotypes presentation | - Limited interpretation and explainability of the AI-model results |
| 1. Learn from data to predict the behavior of the analyzed system | - More accurate quantitative measures to evaluate the utility and privacy preservation are needed |
| 1. Identification of patterns and temporal trends of the patterns (temporal dynamics) | - Insufficient validation for clinical practice |
| 1. Different applications in the multimorbidity research field | - High error-susceptibility |
| 1. Involvement of an expert figure, mostly in the result interpretation phase | |

ability of AI to reveal potential latent variables/factors and time trends within the data, irrespectively of data structure. Indeed, problems associated with multimorbidity have a complex data structure, arising from multiple and overlapping disorders, causing factors, biological determinants, and external sociodemographic variables, in addition to the multiple interactions over time that drive the internal dynamics of multimorbidity (Alsaleh et al., 2023; Hassaine et al., 2020). Hassaine et al. (2020) stressed that most of the previous evidence on multimorbidity has been cross-sectional, which makes them unsuitable for studying and characterizing how a disease progresses over time, while considering how the trajectory interacts within a broader context (*e.g.*, patient's medical history, drug treatments, and presence of multiple disorders) can be very informative. Yet, previous investigations have been focused on a reduced subset of conditions and small samples sizes, hampering their ability to capture the disease clusters and phenotypes. A model to predict multimorbidity has been developed by using a ML approach (with random forest based classifiers) (Polessa Paula et al., 2022). According to this model, using a small set of features and parameters that are easy to collect in clinical practice – BMI, blood pressure, sex, age – is possible to predict multimorbidity. To encourage its use, a web application is also available online,[1] wherein prediction models can be used practically and intuitively by the general public, since it does not require prior knowledge of ML or programming. Altogether, AI technologies can be useful to identify multimorbidity clusters and their evolution over time, as well as patients at risk of progression into multimorbidity, and could help improving clinical decisions for these patients at every health care level.

### 8.2. AI role in frailty

Frailty, by definition, manifests itself as a multidimensional syndrome, hence the application of AI approaches can offer a potential solution to improve its screening and identification (Ambagtsheer et al., 2020), especially in light of the AI's ability to create systems that can analyze and manage complex information. Previous studies suggest that the use of a machine learning-based predictive model could be useful in

detecting future frailty conditions and a higher hospitalization risk, by using both clinical and socioeconomic variables (Mohanty et al., 2022; Tarekegn et al., 2020). In this regard, Sepúlveda et al. (2022) reported that several biomarkers associated with inflammation, oxidative stress, skeletal/cardiac muscle, and platelet function were identified as promising frailty markers, although none of them alone was sufficient to diagnose and predict the presence of frailty. Considering all these biomarkers together, the application of AI methods can possibly improve the capability to detect frailty both in terms of sensitivity and specificity, given that frailty involves multiple biological systems (Sepúlveda et al., 2022). Yet, despite the significant potential of AI to support frailty identification, this remains poorly investigated in the literature, whereas more research that integrates the aforementioned biomarkers together with clinical, nonmedical information, as well as sociodemographic variables (usually available in the health centers records) is needed and represents a promising method to strengthen frailty diagnosis. In this regard, a first attempt has been conducted by six studies, analyzed in the systematic review by Oliosi et al. (2022), which reviewed state-of-the-art literature dealing with frailty screening through innovative AI-methodologies, such as ML approach. Support vector machine was the most frequently used ML method, which seems to be able to identify several risk factors to predict frailty as well as pre-frail stages (Oliosi et al., 2022). Hence, this first evidence suggests that the potential of applying ML techniques to identify frailty biomarkers is immense. According to AI models, old age, being females, clinical conditions (such as arthritis, hypertension, osteoporosis, and diabetes), high use of healthcare utilization, and adverse health outcomes (such as fractures, prolonged length of hospital stay, and number of hospitalizations) were the most significant predictive variables for the screening outcomes in frail persons. Indeed, these findings confirm that AI techniques should be considered as a valuable clinically practical application in frailty screening, as they can support clinical specialists and foster personalized health (Mohanty et al., 2022). Similarly, ML methods seem to be useful also to predict pre-frailty in middle-aged and older adults, with higher BMI, lower muscle mass, poorer grip strength, balance and quality of sleep, higher levels of distress, breath, and incontinence as relevant factors to be included in targeted health assessments aimed at identifying pre-frailty (Sajeev et al., 2022). Lastly, a new frontier about the frailty management that needs to be mentioned consists in the implementation of AI technologies, namely the development of digital platforms that support care coordination and shared care planning of frail elderly (Kouroubali et al., 2022).

### 9. Conclusions

A great and continuously growing number of biomarkers is reported in the scientific literature, including age biomarkers. A detailed and exhaustive discussion of these latter is outside the possibilities of a single review paper. Instead, we decided to focus on a limited number of them and discussed their established or potential usefulness as biomarkers for frailty and age-associated diseases/multimorbidity. In particular, since frailty and multimorbidity as well as old age are characterized by chronic sterile inflammation, decline in cognitive and physical function, we focused on selected parameters that are involved in inflammaging, mitochondrial dysfunction (and the response that it elicits), neurodegeneration, and sarcopenia/osteoporosis.

The idea that age biomarkers can help identifying frail and multimorbid patients stems from the Geroscience concept that aging and age-related diseases share the same molecular mechanisms, therefore, it is a straight-forward hypothesis that biologically older people are also at risk of being frail and/or multimorbid, and, vice-versa, frail and multimorbid patients are likely biologically older than age-matched healthy people. In this review we have tried to collect evidence or suggestions that support this hypothesis (graphically summarized in Fig. 2) for a selected number of biomarkers of age, even though for some of them (GM, EVs) there is still a lack of sufficient evidence and more studies are

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[1] Web application link: https://danielapaula.shinyapps.io/Multilabel_Tool/
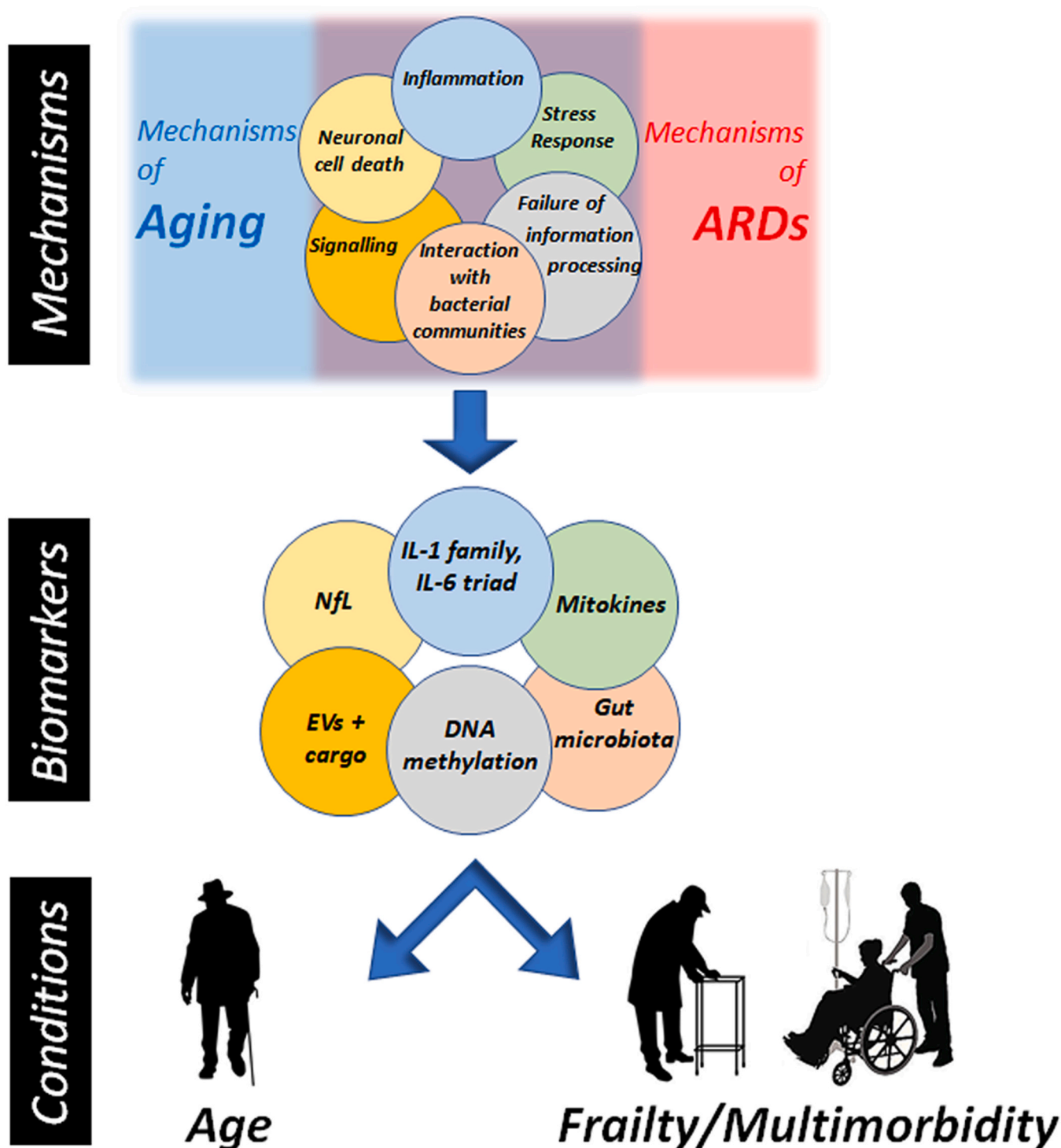
**Fig. 2.** Biomarkers of age can be also biomarkers for frailty and multimorbidity. According to Geroscience, basic molecular mechanisms are shared between aging and age-related diseases (ARD). We have considered in this review some selected biomarkers related to those mechanisms, and discussed available evidence that they can be useful also to identify frail or multimorbid patients.

required. However, many of these biomarkers lack specificity (i.e. many tissues can be a source), and sometimes predictive capacity is not elevated, due to a great interindividual variability, therefore, in order to implement both features, the combination of many biomarkers should be pursued, as mentioned in the introduction. To further increase the diagnostic/prognostic power of composite biomarker panels, the inclusion in each panel of epigenetic regulators such as miRNAs/IsomiRs (Morsiani et al., 2021; Olivieri et al., 2021a; Olivieri et al., 2017),

circular RNAs (Debès et al., 2023), tRNAs-derived fragments (Yuan et al., 2021) and cell free DNA with different methylation pattern (Fox-Fisher, Shemer and Dor, 2023) should be also considered. In fact, blood-borne, cell-free nucleic acids appear to be very promising in this regard, and some biosensors for miRNA are under implementation (Miti et al., 2020). To this regard, it is to note that for many of these next-generation biomarkers, and in particular those obtained from omics techniques, the normality reference intervals have not yet been

determined, limiting the usefulness of these biomarkers, also in the light of the "FAIR" principles (data should be findable, accessible, interoperable and re-usable). What are the best biomarkers and in what combination can be complicate to determine, as the number of biomarkers (and consequently of their possible combinations) is growing exponentially. AI methods can help in this task, by also adding the possibility to include clinical and socioeconomical parameters, which represent a further layer of complication but, at the same time, a still largely unexplored added value as far as the capability to identify frail and/or multimorbid patients and, even more important, subjects at risk for such conditions before their clinical onset.

An ethical *caveat* should be also considered when studying these age biomarkers, especially in the light of AI implementations. In fact, data obtained measuring these biomarkers can potentially provide sensitive information on every single person subjected to analysis, even routinely. If a combination extracted *post-hoc* from an AI program of simple biomarkers obtained for check-up purposes was in fact predictive of a person's biological age, this could become important information that goes well beyond the scopes and intentions of the prescriber and may thus pose a serious issue of privacy and data availability. This is likely to be something policymakers need to consider in the near future, especially in light of the notion that older biological age equals higher risk of disease, as mentioned. Anonymization, ad hoc informed consent from patients and limitations for off-label use of these data will be likely warranted in order to safeguard patient' rights.

Geroscience has revolutionized the approach to aging and age-associated diseases. Anti-aging therapies are on the verge of clinical practice, however, the subjects (or patients) who would benefit more from this approach and these therapies can be efficiently identified and monitored only by a standardized use of biomarkers of age, likely including (some of) those that have been discussed in this review. At the same time, these age biomarkers may have in many cases the double advantage of being able to identify not only biologically older people, but also frail and multimorbid patients, therefore the bench-to-bedside translation of such biomarkers is going to be one of the medical challenges of the next future.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Data Availability

No data was used for the research described in the article.

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